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UNIVERSITÉ DE MONTRÉAL

CONCEPTION D'UN ADAPTATEUR MOLÉCULAIRE POUR L'IMMOBILISATION DE FACTEURS DE CROISSANCE SUR UN SUBSTRAT DE COLLAGÈNE

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Ce mémoire intitulé :

CONCEPTION D'UN ADAPTATEUR MOLÉCULAIRE POUR L'IMMOBILISATION DE FACTEURS DE CROISSANCE SUR UN SUBSTRAT DE COLLAGÈNE

présenté par : ADDI Cyril

en vue de l'obtention du diplôme de : Maîtrise ès sciences appliquées

a été dûment accepté par le jury d'examen constitué de :

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RÉSUMÉ

Les biomatériaux à base de collagène ont une place particulièrement importante dans le domaine de l'ingénierie tissulaire. Leur fonctionnalisation peut être facilement effectuée à l'aide de protéines chimères constituée d'une biomolécule, typiquement un facteur de croissance, fusionnée à un domaine de liaison au collagène. Cette fonctionnalisation peut conférer aux biomatériaux des propriétés mitogéniques et anti-apoptotiques, et influencer la colonisation de ce dernier par des cellules. De nombreuses études ont été menées en ce sens dans des domaines variés de la médecine régénératrice, comme la cicatrisation ou la régénération osseuse.

Nous avons choisi d'immobiliser des facteurs de croissance étiquetés avec une hélice alpha (Ecoil-GF) sur un substrat de gélatine en utilisant un adaptateur moléculaire constitué du domaine de liaison au collagène de la fibronectine, étiqueté avec l'hélice alpha partenaire (CBD-Kcoil). Les hélices alpha Ecoil et Kcoil interagissent par interaction superhélice d'une manière spécifique avec une forte affinité, ce qui permet la formation d'un complexe entre la gélatine, l'adaptateur moléculaire CBD-Kcoil et le facteur de croissance Ecoil-GF. Nous avons produit et purifié le facteur de croissance des fibroblastes basique étiqueté avec l'hélice Ecoil (Ecoil-bFGF), et nous avons comparé son immobilisation avec celle du facteur de croissance épidermique, en utilisant l'adaptateur moléculaire CBD-Kcoil.

ABSTRACT

Collagen-based biomaterials have attracted a lot of interest in the field of tissue engineering. Their functionalization can be easily performed using chimeric proteins composed of a biomolecule, typically a growth factor, that is fused to a collagen-binding domain. This functionalization may provide biomaterials with mitogenic and anti-apoptotic properties, and influence the cellular fate within the implant. Many studies have been conducted to this end, in various fields of regenerative medicine, such as wound healing and bone regeneration.

We chose to tether coil-tagged growth factors (Ecoil-GF) on a gelatin substrate using a molecular adaptor consisting of the collagen-binding domain of fibronectin, fused to the complementary coil (CBD-Kcoil). E and K coils interacted through coiled-coil interaction in a specific manner with high affinity, which enabled the formation of a ternary complex between the gelatin substrate, the molecular adapter CBD-Kcoil and the growth factor Ecoil-GF. We have produced and purified an Ecoil-tagged basic fibroblast growth factor (Ecoil-bFGF), and we have compared its tethering with that of the epidermal growth factor, using the molecular adaptor CBD-Kcoil.

TABLE DES MATIÈRES

LISTE DES TABLEAUX

LISTE DES FIGURES

LISTE DES SIGLES ET ABRÉVIATIONS

aFGF et FGF-1	acidic fibroblast growth factor
bFGF et FGF-2	basic fibroblast growth factor
BCA	bicinchoninic acid assay
BMP	bone morphogenetic protein
CBD	collagen-binding domain
ColH and ColG	collagenase H and G
DBM	demineralized bone matrix
DMEM	Dulbecco's modified eagle medium
EBM	endothelial basal medium
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EPC	endothelial progenitor cells
FBS	fetal bovine serum
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FN	fibronectin
GPI	glycosylphosphatidylinositol
HBS-EP	HEPES buffered saline avec EDTA et Surfactant P20
HGF	hepatocyte growth factor
HUVEC	human umbilical vein endothelial cells

HRP	horseradish peroxidase
IGF	insulin-like growth factor
IPTG	isopropyl β-D-1-thiogalactopyranoside
GF	growth factor
MBM	mineralized bone matrix
MMP	matrix metalloproteinase
MSCs	mesenchymal stem cells
NGF	nerve growth factor
OC	osteocalcin
PBS	phosphate buffered saline
PCL	polycaprolactone
PDGF	platelet-derived growth factor
PlGF	placental growth factor
PTH	parathyroid hormone
SDF-1α	stromal cell-derived factor 1α
SPR	surface plasmon resonance
TEV	tobacco etch virus
TGFβ	transforming growth factor β
TRX	thioredoxin
VEGF	vascular epidermal growth factor
VSMC	vascular smooth muscle cells
vWF	von Willebrand factor

CHAPITRE 1 INTRODUCTION

Le collagène est un matériau très populaire dans le domaine de l'ingénierie tissulaire, en particulier à cause de sa faible antigénicité et de son excellente biocompatibilité, ce qui en fait un matériau très sûr d'utilisation. Le collagène est une protéine fondamentale de la matrice extracellulaire, qu'on retrouve sous la forme d'une trentaine de paralogues, dont le collagène de type I, la protéine la plus abondante du corps humain (Figure 1.1). Il est exprimé dans la majorité des tissus comme la peau, les tendons, les ligaments, le cartilage, les os, les vaisseaux sanguins ou encore les muscles et confère une résistance mécanique aux tissus. Au vu de son importance, c'est une protéine extrêmement conservée au cours de l'évolution (97 % d'identité entre Homo *sapiens* et Bos *taurus*). Cela permet en particulier d'utiliser du collagène extrait de bœuf ou de porc pour fabriquer des biomatériaux qui présentent une bonne biocompatibilité chez l'homme. Un certain nombre d'entre eux sont déjà approuvés par les autorités de santé (la Food and Drug Administration et l'Agence Européenne des Médicaments).

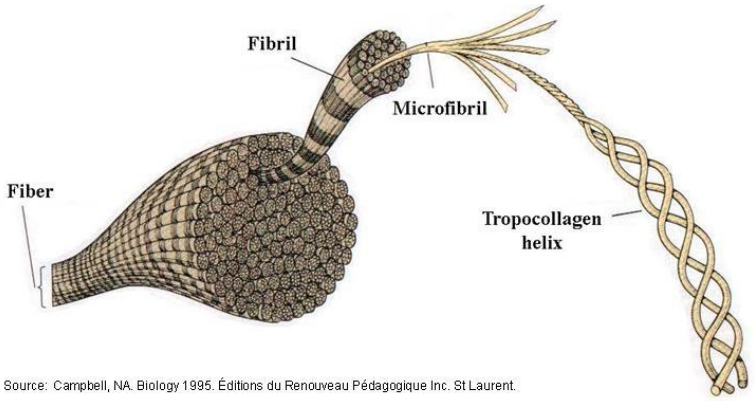

Figure 1.1. Structure du collagène de type I dans le corps humain

Une approche prometteuse a été développée ces dernières années pour fonctionnaliser le collagène et lui donner, par exemple, des propriétés bio-inductives promouvant la migration, la prolifération, ou encore la différenciation cellulaire. Cette approche consiste à développer des protéines de fusion entre un domaine de liaison au collagène (issu de protéines qui se lient

naturellement au collagène, comme la fibronectine ou le facteur de von Willebrand) et un domaine bioactif (par exemple un facteur de croissance ou un domaine de liaison aux intégrines). Ces protéines chimères possèdent donc des propriétés de liaison au collagène en plus de leurs propriétés bio-inductives. De nombreuses études ont montré que ces protéines présentent un pouvoir de régénération beaucoup plus important que leur homologue d'origine (par exemple le facteur de croissance naturel), lorsqu'elles sont utilisées en adéquation avec un biomatériau à base de collagène. En particulier, leur biodisponibilité est augmentée car leur diffusion est limitée : ces protéines chimères peuvent en effet se lier au collagène endogène lorsqu'elles sont libérées du biomatériau. Elles ont prouvé leur utilité dans des domaines très variés de la médecine régénératrice (cicatrisation, régénération osseuse et neurale).

La production de ce type de protéines de fusion (Figure 1.2.A) peut être délicate, dans la mesure où l'ajout d'un domaine de liaison au collagène (CBD) à un facteur de croissance peut altérer le repliement de ce dernier, et affecter sa bioactivité.

L'objectif de ce travail est de développer une stratégie de fonctionnalisation du collagène basée d'une part sur un adaptateur moléculaire constitué du CBD de la fibronectine, fusionné à une hélice alpha (Kcoil), et d'autre part sur des facteurs de croissance (GF) étiquetés avec l'hélice alpha complémentaire (Ecoil). L'interaction superhélice E/K étant une interaction de très forte affinité, un complexe peut se former entre le collagène (ou la gélatine), l'adaptateur moléculaire (CBD-Kcoil), et les facteurs de croissance (Ecoil-GF) (Figure 1.2.B). Cette approche peut théoriquement être appliquée à n'importe quel biomatériau obtenu à partir de collagène.

La première partie de ce mémoire présente une revue critique de la littérature sur les protéines chimères contenant un CBD. En particulier, les différents CBD reportés dans la littérature sont comparés et caractérisés, et les avantages des protéines chimères développées dans les domaines de la médecine régénératrice sont mis en évidence.

La seconde partie présente l'ensemble de la démarche de fonctionnalisation d'une surface de gélatine, en immobilisant des facteurs de croissance à l'aide d'un adaptateur moléculaire. Elle compile l'ensemble des résultats d'expériences, depuis la production et la purification des protéines de fusion, jusqu'à la caractérisation des interactions en jeu dans la fonctionnalisation de surface, et la réponse cellulaire engendrée.

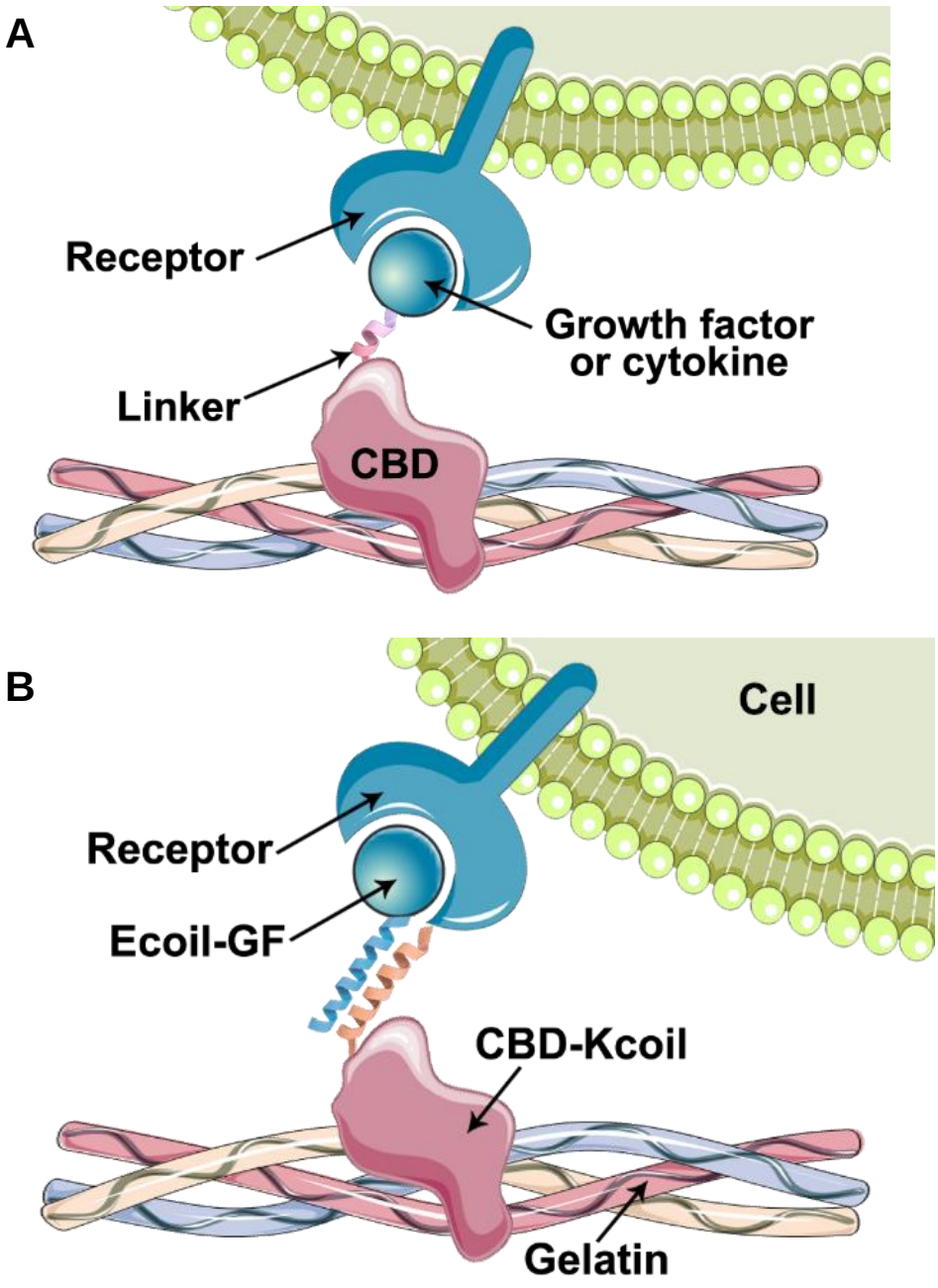

Figure 1.2. Stratégie de fonctionnalisation du collagène à l'aide d'une seule protéine chimère (A) ou de deux protéines interagissant par interaction superhélice (B).

CHAPITRE 2 DÉMARCHE GÉNÉRALE

Le cœur de ce mémoire de maîtrise est composé de deux articles. Le premier, intitulé « The design and use of chimeric proteins with a collagen binding domain for tissue engineering and regenerative medicine » a été soumis à *Advanced Drug Delivery Reviews* en juillet 2016. Cet article compile l'ensemble des domaines de liaison au collagène reportés dans la littérature et compare leurs propriétés biophysiques. Les connaissances développées dans cette revue permettent de mieux situer le CBD employé au Chapitre 3 (issu de la fibronectine) par rapport aux autres CBD. En particulier, elles montrent que ce dernier a été employé depuis plusieurs années, seul ou sous forme de protéine de fusion, qui ont été testées *in vivo* avec succès. Cela démontre le grand potentiel de ce dernier, aussi bien au niveau de ses caractéristiques biophysiques (haute affinité pour le collagène) que de sa biocompatibilité (étant donné qu'il est dérivé de la fibronectine humaine).

Le second article, intitulé « The use of a chimeric collagen binding domain of fibronectin to recruit coil-tagged growth factors on gelatin-based biomaterial », a été soumis à *Acta Biomaterialia* en juin 2016. Ces deux journaux scientifiques ont été sélectionnés pour leur niveau d'impact dans le domaine étudié ; *Acta Biomaterialia* étant un journal de référence concernant la fonctionnalisation de biomatériaux dans le domaine de la médecine régénératrice tandis que *Advanced Drug Delivery Reviews* publie des revues de littérature sur les techniques émergentes dans le domaine des stratégies thérapeutiques.

J'ai réalisé moi-même l'essentiel des travaux expérimentaux, avec l'aide et le soutien de Frédéric Murschel, Benoît Liberelle et Nesrine Riahi. J'ai aussi bénéficié de l'expérience de mon directeur de recherche Gregory De Crescenzo et de Frédéric Murschel concernant l'avancement général du projet de recherche et tout particulièrement pour la relecture des articles.

CHAPITRE 3 REVUE DE LITTÉRATURE

De nombreuses techniques ont été développées pour fonctionnaliser les biomatériaux avec des molécules bioactives afin d'influencer la migration, la prolifération ou encore la différenciation cellulaire. Parmi toutes ces approches, la création de protéines de fusion comprenant un CBD est particulièrement prometteuse pour fonctionnaliser les biomatériaux à base de collagène. De nombreuses protéines, comme des facteurs de croissances, ont ainsi été fusionnées à un CBD pour augmenter leur biodisponibilité, avec souvent un effet très bénéfique, y compris lors d'études *in vivo*. Une revue de l'ensemble des travaux reportés dans ce domaine est présentée ici dans un manuscrit récemment soumis à *Advanced Drug Delivery Reviews*.

3.1 Article 1 – The design and use of chimeric proteins containing a collagen binding domain for tissue engineering and regenerative medicine

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3.2 Abstract

Collagen-based biomaterials are widely employed in the field of tissue engineering; they can be loaded with biomolecules such as growth factors to modulate the biological response of the host and thus improve their potential of regeneration. Recombinant chimeric growth factors fused to a collagen-binding domain have been reported to improve the bioavailability of these growth factors, especially when combined to an appropriate collagen-based biomaterial. This review first provides an extensive description of the various collagen-binding domains that have been characterized and fused to several proteins for application in the fields of tissue engineering and regenerative medicine. The second part of the review highlights the benefits of various collagen-binding domain /growth factor fusion proteins that have been designed for wound healing and bone regeneration.

Keywords— growth factor; collagen-binding domain; collagen; gelatin; biofunctionalization; scaffold; coating; delivery

3.3 Introduction

Collagen has attracted a lot of attention in the field of tissue engineering, given its excellent biocompatibility, biodegradability, weak antigenicity and safety[1]. Biomaterials made of collagen have been commonly used in various formulations, such as hydrogels, sponges and microparticles; they are often made of denatured collagen (i.e. gelatin)[2]. Importantly, some of them are already approved by the Food and Drug Administration[3]. Moreover, the mechanical properties of collagen scaffolds can be tuned by cross-linking, resulting in higher tensile strength and proteolytic resistance[4], [5] and allowing for their use in a wider scope of applications. Also, in order to improve their healing potential, these biomaterials have been loaded with various biomolecules to be released over time, be it small molecules such as steroids, antibiotics and chemotherapy agents, proteins such as growth factors and antibodies, or liposomes[4]. The potential benefits of their loading with biomolecules are numerous as it enhances effective local concentrations of drugs,

hence preventing side effects due to systemic distribution and it increases drug half-life in many cases[6].

Most of the collagen-derived biomaterials that have been developed for tissue engineering/regenerative medicine purposes simply trap the drug in their network and release it over time by diffusion through the pores of the scaffold[6]. In this delivery strategy, the difficulty to control drug release represents a major drawback: there is generally an initial burst release which is not followed by a sustained release over a long period[7], [8]. In addition to the rapid decrease of drug concentration in time, the initial burst itself may be an issue. For example, in the case of Bone Morphogenetic Protein-2 (BMP2) release, it was shown to promote inflammation and osteoclastic activity[7]. Conversely, reducing the size of the pores in order to extend the release hinders nutrient transport and thus severely affects cell viability[9].

Growth factors are frequently employed in therapeutic strategies such as bone and cartilage engineering[10], myocardial regeneration[11] and wound healing[12] because they can promote cell adhesion, migration, differentiation and proliferation. The optimal beneficial effect of these growth factors is determined by their spatiotemporal delivery, an essential trait that can affect cell fate[13]. Although no growth factor binding site has been reported in collagen yet[14], some growth factors, e.g. Transforming Growth Factor-β1 (TGF-β1), basic Fibroblast Growth Factor (bFGF) and BMP2, have a naturally strong affinity for collagen and stably interact with scaffolds made of collagen through ionic interactions[7], [15], [16]. However, these interactions mainly depend on the isoelectric point of the growth factor[7], [17] and the release profile is often characterized by an initial burst[7]. Therefore, it does not constitute the optimal manner to control the release of growth factors.

A variety of techniques have been developed for the covalent chemical conjugation of growth factors to collagen scaffolds so as to provide a highly localized and long-lasting signaling. The random covalent grafting of growth factors has proved worthwhile[18], [19], in particular through the NHS/EDC coupling chemistry that targets free amine groups on the growth factor[20] or by photo-irradiation[21]. However, the bioactivity of the immobilized proteins may be negatively impacted, since their attachment to the substrate may mask or alter their receptor binding moieties[22], [23]. The covalent binding of growth factors in an oriented manner with the help of

a cysteine tag has also been investigated[24]. However, the addition of a cysteine can hamper protein folding via the formation of unwanted disulfide bridges, hence resulting in a loss of bioactivity. Importantly, the covalent binding of growth factors may also prevent their endocytosis, which can be crucial for signaling[25].

The tethering of growth factors in a stable but non-covalent manner through specific affinity tags has also been extensively studied. The major strategies that have been tested include the interactions between biotin and streptavidin[26], coil peptides[27]–[29], DOPA-containing peptides[30] as well as the use of binding domains for heparin/heparan sulfate[31], laminin[32], fibrin[33] or fibronectin[34]. Among them, the use of several collagen-binding domain (CBD) fusion proteins in association with collagen-based biomaterials has been the subject of intense research. Indeed, type I collagen is the most abundant protein in the human body[35]; it is found in the extracellular matrix of bones, skin, tendons, cornea, artery walls as well as internal organs. Hence, a recombinant collagen-binding growth factor being tethered to a resorbable collagen biomaterial would also bear the potential to bind to the extracellular collagenous matrix after resorption of the implant, which would significantly improve its bioavailability over time.

The purpose of this review is (i) to examine the various CBDs that have been characterized and discuss how their origin and size affect their affinity and specificity for collagen, and (ii) to assess to which extent collagen biomaterials functionalized with CBD fusion proteins have emerged as powerful tools in the fields of wound healing and bone regeneration.

3.4 Collagen-binding domains

The term collagen-binding domain (CBD) encompasses very distinct polypeptide domains that are either engineered or derived from native collagen-binding proteins such as fibronectin, the von Willebrand Factor and several collagenases. This diversity of origin results in a great heterogeneity of sizes, as they range from a seven amino-acid long peptide to a whole protein domain of 42 kDa (Table I). Bearing in mind that these domains will be fused to growth factors and that controlled release is critical in tissue engineering and regenerative medicine, it is crucial to assess how their sequence and their length affect their interaction with collagen, especially in terms of stability.

3.4.1 Quantitative characterization of CBD-collagen interactions

For most of the CBDs studied in the literature, the strength of their interaction with collagen has been determined, either by reporting an apparent affinity, or thermodynamic association constant (K_A, expressed in M^{-1}), or its inverse, the thermodynamic dissociation constant (K_D, expressed in M). A plethora of dissociation constant values, spanning from the low nanomolar to the micromolar, have been determined for the various CBD-collagen interactions (see Table I). These differences in apparent affinities may not be surprising given the diverse origins of the CBDs and types of collagen. Such a range of affinities may be exploited to target specific types of collagen by selecting an appropriate CBD or to modulate the release of a given protein fused to a specific CBD by virtue of its affinity for collagen. However, in order to avoid any misinterpretation of the data presented in Table I, one may analyze them with caution.

Many techniques have indeed been employed by the research community to determine the affinities of the various CBDs for the different types of collagen. Those include Surface Plasmon Resonance (SPR)-based biosensor assays[36], Enzyme-Linked Immunosorbent Assays (ELISA)[14], radioactivity assays by using iodinated proteins[37] as well as fluorescence titration assays by measuring changes in anisotropy of fluorescein-labeled collagen chains[38]. All of these methods have their own merits and limitations to assess an apparent dissociation constant[39]. Interestingly, large differences can be pinpointed between the values derived from distinct techniques within the same report. For example, when characterizing the interactions between the von Willebrand A1 domain for type III collagen, Morales et al. determined an apparent K_D of 8 nM by SPR and of 400 nM by ELISA[40]. Such a discrepancy can result from several biases inherent to the techniques at hand: in most SPR assays, the collagen is covalently attached to the biosensor surface, whereas, in ELISA, the substrate is non-specifically adsorbed at the bottom of the well. Both approaches may lead to distinct alterations of the three-dimensional conformation of the triple helix of collagen and, as a result, change the strength of its interaction with CBDs[40]. Other artefacts related to inappropriate assay conditions, as those documented in specialized reports[41], [42] may also be invoked to explain these discrepancies.

It is also not uncommon that distinct research groups report different apparent K_D for the same interaction, even when assessed with the same assay (see Table I). For example, Sun et al. reported

an apparent K_D of 5.5 nM for the capture of the fusion protein TKKTLRT-PDGF (platelet-derived growth factor) by a type I collagen membrane, whereas Lin et al. reported an apparent K_D of 97 nM for the same protein incubated on type I collagen-coated wells[43], [44]. As much as the techniques, the procedure for data processing and K_D determination may therefore be worth questioning. In most of the articles here reviewed, the apparent K_D values were indeed derived by linearizing the experimental data, via a Scatchard plot for instance. Such an approach is prone to introduce errors and biases[45], especially when the plot deviates from linearity and when data are omitted[46], [47].

To overcome these limitations, we here propose a rationalized method for data processing. By curve-fitting the complete data set with the GraphPad Prism 6 Software (GraphPad Prism Software Inc., San Diego, CA), using a Hill-type equation that assumes a 1:1 interaction, the half-maximum effective concentration (EC_{50}) of a CBD binding to collagen can be calculated:

$$Y = \frac{Y_{max}}{1 + \frac{EC_{50}}{[CBD]}}$$

Where [*CBD*] and *Y* correspond to the incubated CBD concentration and the corresponding response (signal minus blank), respectively. Y_{max}, the theoretical maximal response, and EC_{50}, the half-maximum effective concentration, are outputs. Note that the Hill slope was fixed at 1.0 for the sake of comparison.

This curve-fitting method was applied to all the CBD-collagen interaction studies, when raw results were available, by extracting the data from the research article figures using the WebPlotDigitizer 3.9 software[48]. When applicable, the EC_{50} values derived from the fits, the coefficient of determination (R^2) as well as the number of experimental data points extracted from the figures (*n*) are given in Table I, in addition to the apparent dissociation constant value (K_D) that was reported by the authors. Although all R^2 values were close to 1, which is indicative of a good fit between the model and the data, some research groups have used a low number of CBD concentrations in their investigation. Given that the reliability of any curve-fitting is highly dependent on the number of experimental points, both the K_D values they reported and the EC_{50} values we calculated need

to be treated with caution. Furthermore, we encountered several cases of biphasic interactions, for which we provided only the lowest EC_{50} (corresponding to the highest apparent affinity).

With this analytical approach, we found that the interaction of TKKTLRT-PDGF for type I collagen, with reported K_D values of 5.5 nM and 97 nM by Sun et al. and Lin et al., respectively, was best depicted by an EC_{50} of 1 100 nM in both cases[43], [44]. This calculated value is plausible, given that the majority of the TKKTLRT fusion proteins have a dissociation constant in the low micromolar range (Table I).

3.4.2 Fibronectin

Fibronectin (FN) is a 220-kDa protein of the ECM which exists as a dimer and binds to many biological partners such as collagen, fibrin, heparin and integrins (Fig. 1A). FN has a very high apparent affinity for the type I (K_D = 13 nM)[49] and type II (K_D = 58 nM) collagens[50], and it binds even more strongly to their denatured form, gelatin[51], (K_D = 2 nM)[50], which suggests that the binding site(s) within collagen are, at least partially, masked in the native triple helix[49], [52].

The CBD of FN is a 42-kDa domain composed of six modules (I_6-II_1-II_2-I_7-I_8-I_9, see Fig. 1A) that are all required for full affinity[53], supporting a cooperative mode of interaction where all modules simultaneously participate[54], [55]. The FN type II modules are found in many other proteins, including the mannose receptor[56], the Factor XII[57] and the matrix metalloproteinase 2 (MMP2)[55], providing them with collagen-binding ability. Similar to the full-length FN, its CBD displays a higher affinity for gelatin than for properly folded collagens. The CBD of FN is however monomeric and has a moderate affinity for type I collagen (K_D = 420-800 nM)[49], [58], hence highlighting the importance of FN dimerization (and of the resulting avidity) for optimal binding to collagen. Numerous subdomains containing three or more modules have been studied, including a short peptide sequence extracted from the module I_9, CQDSETRTFY, which binding to type I collagen proved to be very weak (EC_{50} = 900 000 nM, Table I)[59], [60].

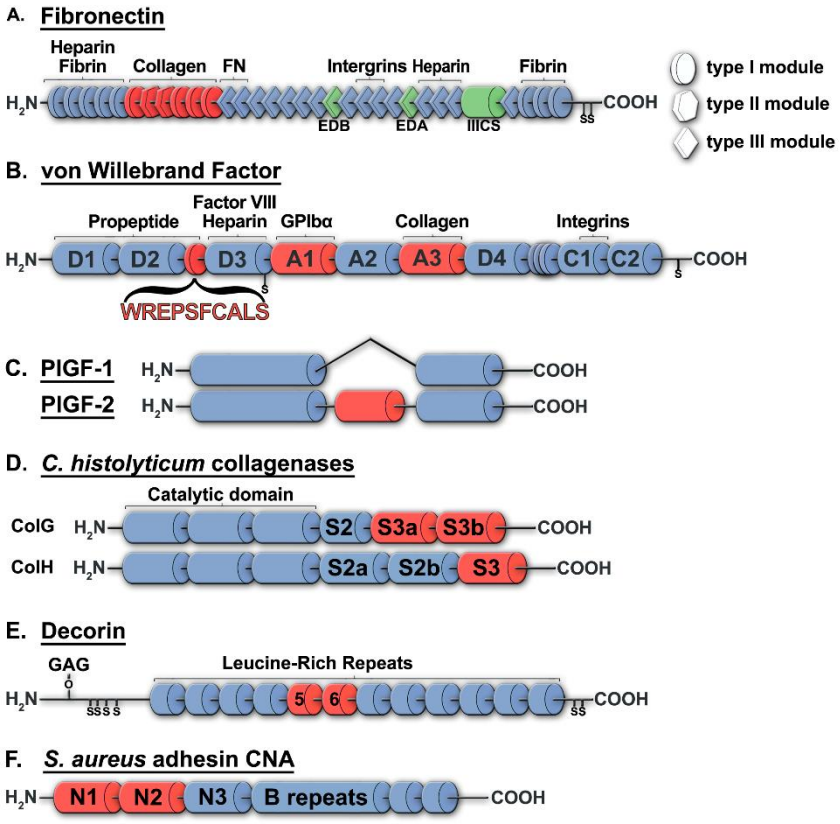

Figure 3.1. Structure of several proteins which contains a collagen-binding domain (in red): human fibronectin (A), von Willebrand Factor (B), placental growth factor (C), *C. histolyticum* collagenases ColG and ColH (D), decorin (E) and *S. aureus* adhesin (F).

3.4.3 Von Willebrand Factor

The von Willebrand Factor (vWF) is a 275-kDa blood glycoprotein involved in hemostasis that is frequently encountered as a multimer (due to disulfide bonds), which improves its hemostatic potential[61]. The mature vWF has a very high affinity for type I (K_D = 1.8 nM)[62], type III (K_D = 3.4 nM)[63] and type VI (K_D = 8 nM)[64] collagens. It poorly binds to denatured collagen, in stark contrast to FN[65], [66]. Several moieties of the vWF have been studied for their ability to bind to collagen (Fig. 1B), in particular the A1 (20.3 kDa) and A3 domains (19.3 kDa)[67]. Although conflicting results exist in the literature, the A1 domain of vWF does not seem to play a

significant role in the binding of vWF to type I/III collagens[68], [69] but is crucial for its interaction with the type IV/VI collagens[64], [70], [71]. On the contrary, the A3 domain is considered to be the main collagen-binding site of the vWF[72], [73] and have a moderate affinity for the type I (K_D = 1 800 nM)[67] and type III collagens (K_D = 1 600 nM)[40]. The A3 domain is thought to interact with the RGQAGVMGF/RGEOGNIGF sequences of the α1/α2 chains of type I collagen and with the RGQOGVMGF sequence in type II and III collagens[69]. Shorter peptide sequences, derived from the bovine and human vWF have also been studied: WREPSFCALS and WREPGRCELN, respectively (Fig. 1B)[37]. These short peptides have often been mutated, that is, their cysteine residue has been replaced by a methionine (WREPSF[M]ALS or WREPGR[M]ELN)[74], [75] in order to avoid unwanted homodimerization or misfolding[76], [77]. The WREPSFCALS peptide is derived from a 21-kDa sequence (F^{570} - K^{682}) of the bovine vWF (NP_001192237.1), described by Takagi et al.: this sequence is located at the end of the vWF propeptide[78] which is cleaved during posttranslational modifications of vWF[79]. As opposed to the A3 domain, the affinity of the WREPSFCALS peptide for type I collagen is very weak (EC_{50} = 29 000 nM, Table I)[37]. Nonetheless, it binds to all collagen types from I to V, in addition to gelatin[37]. Its 21-kDa parent fragment possesses the same characteristics but displays a higher affinity for type I collagen (EC_{50} = 780 nM, Table I)[37].

3.4.4 Placental Growth Factor

Another protein from which the potential of its short CBD has been recently highlighted is the Placental Growth Factor (PlGF, 16.7 kDa), a member of the VEGF family, that is essential for angiogenesis, in particular over the bone-marrow derived cells[80]. The sequence analysis of two splice variants of PIGF which interact differently with the ECM, PlGF-1 and PlGF-2, allowed the identification of a 2.8-kDa amino acid sequence that binds to collagen (RRRPKGRGKRRREKQRPTDCHL, Fig. 1C)[14]. Indeed, while initially considered as a heparin binding domain[81], this sequence binds very strongly to type I collagen (K_D = 126 nM) and even more to Fibronectin, Vitronectin, Heparan Sulfate and other ECM proteins[14]. Although this sequence has a very high isoelectric point (pI = 12.0), electrostatic interactions only are not sufficient for collagen binding since a scrambled version of this peptide does not bind to type I collagen[14]. Conversely, Martino and colleagues assessed that the cysteine at the C-terminus of

the sequence can be mutated to a serine with little to no influence on the interaction with type I collagen[14]. No further experiment has been reported yet concerning the specificity of this CBD towards the other collagen types.

3.4.5 C. histolyticum collagenases

ColH (116 kDa) and ColG (126 kDa) are two collagenases from the pathogenic *Clostridium histolyticum* (Fig. 1D)[82]. They feature a broad substrate specificity and target various types of collagens as well as gelatin[83], [84]. For instance the full-length ColH enzyme displays a strong affinity for type I collagen (K_D = 99.5 nM)[85]. Their CBD, corresponding to the S3 domain (13 kDa) for ColH and the S3a+S3b domains (26 kDa) for ColG, are often associated with the polycystic kidney disease-like domain that precedes them, that is S2a+S2b (20 kDa) for ColH and S2 (10 kDa) for ColG (Fig. 1D).

The fragment corresponding to the S2b+S3 domains of ColH displays a biphasic interaction with collagen, presenting a strong affinity (K_D = 339 nM) followed by a moderate affinity at higher concentrations (K_D = 2110 nM)[85], whereas the S3 domain alone poorly binds to type I collagen (K_D = 15 900 nM)[85].

The short fragment of ColG corresponding to the S3a+S3b domains (Fig. 1D) binds to all collagen types from I to IV, and even $(POG)_n$ (K_D = 63 000 nM)[86], where $(POG)_n$ is a collagen-like peptide, provided that the number of repeats, *n*, is large enough to allow the peptide to have a triple-helical conformation[87]. Likewise, it does not bind to gelatin, suggesting that this CBD recognizes the triple-helical structure of collagen[84], [86].

3.4.6 Decorin

Decorin is a small proteoglycan which interacts with collagen fibrils in all connective tissues[88, pp. 4–5]. Similarly to biglycan and fibromodulin, its core protein is mainly constituted of leucine-rich repeats (LRR). Although the glycosaminoglycan chains play a role in the binding of decorin to collagen, the core protein alone also binds to all types of collagen (I-VI) with strong affinity (K_D = 6 nM for type I collagen)[89]–[91]. Its CBD is located in the LRR 5-6[88], [89] of the core protein (NP_001911.1, Fig. 1E), it binds to type I collagen with moderate affinity (EC_{50} = 7 400

nM, Table I)[89]. This CBD contains a small sequence SYIRIADTNIT that is, when used as a single peptide, is able to specifically inhibit the interaction between decorin and type I collagen (K_i = 4 000 nM)[89].

3.4.7 *S. aureus* adhesin

Staphylococcus aureus is a very common opportunistic pathogen which persistently colonizes about 20% of the human population[92]. As a Gram-positive bacterium, *S. aureus* is covered with adhesive proteins, among which are adhesins[93]. CNA35 is a 35-kDa fragment of the *S. aureus* adhesin (CNA) that hooks the bacteria to collagen. This CBD is composed of two domains: N1 and N2, that can entwine the collagen triple helix (Fig. 1F)[94]. The interaction of CNA35 with the type I collagen shows a biphasic behavior characterized by a high affinity binding mode (K_D = 500 nM) and a low affinity component of binding (our analysis indicated an EC_{50} > 300 000 nM)[95]. To a lower extent, this CBD also binds to collagen II, III and IV, but neither to collagen V nor to collagen VI[95].

3.4.8 *V. mimicus* metalloprotease

Vibrio mimicus is a pathogenic bacterium which is responsible for certain cases of gastroenteritis. It expresses a metalloprotease named VMC, which CBD, LVLSRPGQFAQWAQT VKNLGEQYNAEFAVWLDT (3.8 kDa), contains two FAXWXXT repeats shown to be very important for its collagen-binding ability[96]. In particular, the second repeat is very conserved in several species of *Vibrio*[96]. This VMC metalloprotease targets type I, II and III collagens in addition to gelatin[97]. However, the 33 amino-acid-long CBD has a moderate affinity for type I collagen (K_D = 4 000 nM)[96].

3.4.9 Engineered collagen-binding peptides

Another well-studied CBD that corresponds to the peptide sequence TKKTLRT has been engineered to be the antisense peptide of the collagenase-cleavage site within the α2 chain of type I collagen[98]. This peptide has almost the same hydrophilicity plot as SQNPVQP and SSNPIQP which are part of the matrix metalloproteinase 1 (MMP1) and the neutrophil collagenase, respectively[98]. TKKTLRT has a moderate affinity for type I collagen (our analysis indicated an

EC_{50} equal to 2300 nM, Table I)[98] and has the ability to bind to gelatin[99]. However, Fukata and colleagues demonstrated that its maximal binding capacity is significantly weaker than that of the fragment corresponding to the S2b+S3 domains derived from the *C. histolyticum* ColH[100]. Although its isoelectric point is very high (pI = 11.2), this characteristic is not sufficient for its optimal interaction with collagen as De Souza and colleagues demonstrated that a scrambled version (LTTTKKR) did not bind to collagen[98].

Other collagen-binding peptides were identified by phage display[101] or by ribosome display, an in vitro method for selection and evolution of peptides (unpublished results)[102].

3.4.10 Collagen-mimetic peptide

The collagen-mimetic peptide, also known as collagen hybridizing peptide, is composed of 6-10 repeats of the sequence GPO (or GPP), where O is the hydroxyproline[103]. Since this peptide mimics collagen triple helix conformation, it has a high propensity to hybridize to collagen both in vitro and in vivo[103]. More particularly, it targets denatured collagen since it interacts with unfolded collagen strands to form a triple helical structure[104]. Collagen is frequently denatured in pathological conditions such as cancer, atherosclerosis, arthritis and fibrosis; the collagen-mimetic peptide can thus be employed to target the denatured collagen for therapeutic application in this context[101]. It has demonstrated high specificity and high affinity for type I-V collagen, displaying a dissociation constant close to 10 nM, depending of the number of the repeats[105]. However, since it spontaneously self-assemble into helical homotrimers, it needs to be heated or deprotected from a photo-cleavable group, in order to be single stranded[101].

Table 3.1. Reported K_D and calculated EC_{50} of various collagen-binding polypeptides.
See paragraph 3.4.1 for our methodological approach.
*: the plateau (saturation) has not been reached during the experiment.

CBD origin			Reported K_D		Calculated EC_{50}			
Protein	**Subunit**	**Substrate**	**SPR (nM)**	**ELISA (nM)**	**EC_{50} (nM)**	**R^2**	**n**	**Reference**
human fibronectin	full-length FN	Col I α1		13	70	0.975	15	Ingham, 1988[49]
		Col I α1			340	0.999	6	Ingham, 2002[38]
		Col II		58	75	0.991	6	An, 2014[50]
		Denat. Col II		2	3	0.980	6	An, 2014[50]
		type A gelatin	0.08					Van Vlierberghe, 2009[106]
		type B gelatin	2					Van Vlierberghe, 2009[106]
		Col IX	3					Parsons, 2011[107]
	I_6-II_1-II_2	Col I α1	31 000					Pickford, 2001[108]
	I_6-II_1-II_2-I_7	Col I			410	0.981	10	Steffensen, 2002[55]
		Denat. Col I			580	0.985	10	Steffensen, 2002[55]
	I_6-II_1-II_2-I_7-HGF	Col I			76*	0.983	6	Kitajima, 2007[109]
	I_6-II_1-II_2-I_7-I_8-I_9	Col I α1		420	610	0.995	13	Ingham, 1988[49]
		Col I		800	750	0.941	10	Ingham, 1989[58]
		Col I α2		1 800	1 200	0.995	28	Ingham, 2002[38]
		$(POG)_{10}$		150 000	120 000	0.986	90	Ingham, 2002[38]
		gelatin		650	800	0.973	14	Katagiri, 2003[53]
	CQDSETRTFY	Col I			900 000	0.997	6	Sistiabudi, 2009[60]
human von Willebrand Factor	full-length vWF	Col I		1.8	2	0.962	10	Pareti, 1986[62]
		Col III	3.4		0.8	0.985	8	van der Plas, 2000[63]
		Col I			8	0.992	8	Vanhoorelbeke, 2000[110]
		Col VI		8	10	0.998	5	Hoylaerts, 1997[64]
		Col VI			3	0.988	8	Vanhoorelbeke, 2000[110]
		Col III			20	0.984	5	Lankhof, 1996[73]
		Col III	2					Li, 2002[36]
		Col I	30					Li, 2002[36]
		Col III peptide	2					Lisman, 2006[69]
	A1 domain	Col I	6					Morales, 2006[40]
		Col III	10	400	430	0.932	6	Morales, 2006[40]
	A3 domain	Col I		1 800	1 800	0.973	7	Cruz, 1995[67]
		Col III	8					Li, 2002[36]
		Col III peptide	1800					Lisman, 2006[69]
		Col III		1 600	2000	0.997	6	Morales, 2006[40]
	EGF-A3	Col I			580	0.998	5	Kato, 2007[111]
	21 kDa fragment	Col I			780	0.971	9	Takagi, 1992[37]
	WREPSFCALS	Col I			29 000*	0.999	5	Takagi, 1992[37]
	WREPSFCALS-bFGF	Col I			1 300*	0.995	8	Andrades, 2001[112]
		Col I			670	0.992	7	Zhao, 2007[113]
Placental Growth Factor 2	$PlGF2_{(123-144)}$	Col I		126	110	0.996	7	Martino, 2014[14]
	BMP2-$PlGF2_{(123-144)}$	Col I		110	100	0.986	7	Martino, 2014[14]
	PDGF BB-$PlGF2_{(123-144)}$	Col I		96	100	0.991	7	Martino, 2014[14]
	VEGF A-$PlGF2_{(123-144)}$	Col I		124	120	0.994	7	Martino, 2014[14]

CBD origin			Reported K_D		Calculated EC_{50}			
Protein	**Subunit**	**Substrate**	**SPR (nM)**	**ELISA (nM)**	**EC_{50} (nM)**	**R^2**	**n**	**Reference**
C. histolyticum Collagenase G	S3b	$(POG)_8$	547 000					Matsushita, 2001[86]
		Col I			12 000*	0.915	10	Fukata, 2014[100]
	S3aS3b	$(POG)_8$	63 000					Matsushita, 2001[86]
		Col I			5 200*	0.976	10	Fukata, 2014[100]
C. histolyticum Collagenase H	full-length ColH	Col I		99.5	100	0.963	15	Matsushita, 1998[85]
	S3	Col I		15 900	22 000*	0.990	17	Matsushita, 1998[85]
		Col I			12 000*	0.985	9	Fukata, 2014[100]
	S2bS3	Col I		339	2 000	0.980	17	Matsushita, 1998[85]
		Col I			2 100	0.934	9	Fukata, 2014[100]
		$(POG)_8$	3					Brewster, 2008[114]
	FGF1-S2bS3	$(POG)_8$	8					Brewster, 2008[114]
S. aureus CNA	N1-N2-N3 CNA55	Col I		2 100	1 800	0.979	7	Xu, 2004[115]
		Col I	1300	210	290	0.997	27	Rich, 1999[116]
		Col I		2200				Zong, 2005[94]
	N1-N2 CNA35	Col I		200				Zong, 2005[94]
		DBS4	3					Zong, 2005[94]
		(GPO)11	140					Zong, 2005[94]
		(GPP)11	7.5					Zong, 2005[94]
		Col I		500	260	0.992	11	Krahn, 2006[95]
		Col I		21	26	0.991	11	Xu, 2004[115]
		Col I	91		17	0.988	6	Kang, 2013[93]
V. mimicus metalloprotease	Full-length VMC	Col I		2100			5	Lee, 2005[96]
	CBD	Col I		4000			4	Lee, 2005[96]
Decorin	full-length Decorin	Col I		6	4	0.977	8	Kalamajski, 2007[89]
		Col I	21					Nareyeck, 2004[91]
		Col VI	39					Nareyeck, 2004[91]
	LRR 5-6	Col I			7400	0.977	8	Kalamajski, 2007[89]
Engineered peptide	TKKTLRT	Col I			2 300	0.980	7	De Souza, 1992[98]
	TKKTLRT-EGF	Col I		239	1 600	0.937	6	Yang 2009[117]
	TKKTLRT-VEGF	Col I		430	2 400	0.963	7	Zhang, 2009[118]
	TKKTLRT-bFGF	Col I			270	0.991	7	Zhao, 2007[113]
	TKKTLRT-BMP2	Col I		270	140	0.999	4	Chen, 2007[119]
		Col I			6 600	0.979	4	Zhao, 2009[120]
	TKKTLRT-NGF	Col I		510	4 500	0.992	4	Sun, 2007[47]
		Col I		510	6 500	0.951	6	Sun, 2010[121]
	TKKTLRT-NT3	Col I		350	850	0.973	7	Fan, 2010[122]
	TKKTLRT-PDGF	Col I		5.5	1 100	0.971	7	Sun, 2007[44]
		Col I		92	1 100	0.991	7	Lin, 2006[43]
	TKKTLRT-PTH	Col I		292	1 200	0.996	7	Wu, 2013[123]
	TKKTLRT-BDNF	Col I		470	1 100	0.995	8	Liang, 2010[124]
		Col I		420	1900	0.944	4	Han Q, 2009[46]
	TKKTLRT-EphA4LBD	Col I		430	1 500	0.990	7	Li, 2016[125]
	TKKTLRT-PlexinB1LBD	Col I		360	2 900	0.995	7	Li, 2016[125]
	TKKTLRT-EGFR Ab	Col I		460	2 800	0.955	8	Liang, 2015[126]
		Col I		210	1 600	0.968	7	Liang, 2016[127]

3.5 Applications of collagen-binding fusion proteins

Numerous recombinant collagen-binding proteins have been reported in the literature, most of which are growth factors or cell-binding domains. The majority of these fusion proteins have been produced in bacteria, as it can yield very high quantity of proteins, although these are not glycosylated. According to the UniProt database, all of the previously described CBDs - except those from fibronectin and decorin - are naturally non-glycosylated, and thus can be produced as recombinant protein in *E. coli* without any loss of collagen-binding activity[55], [67], [128]. In the specific case of FN, it is not clearly established if the glycosylation of the Asn511 residue (within the module I_8) significantly impacts the affinity of this CBD for collagen[129]–[131].

This review will focus on the in vivo applications of these recombinant proteins, especially wound healing, bone regeneration and neuroregeneration.

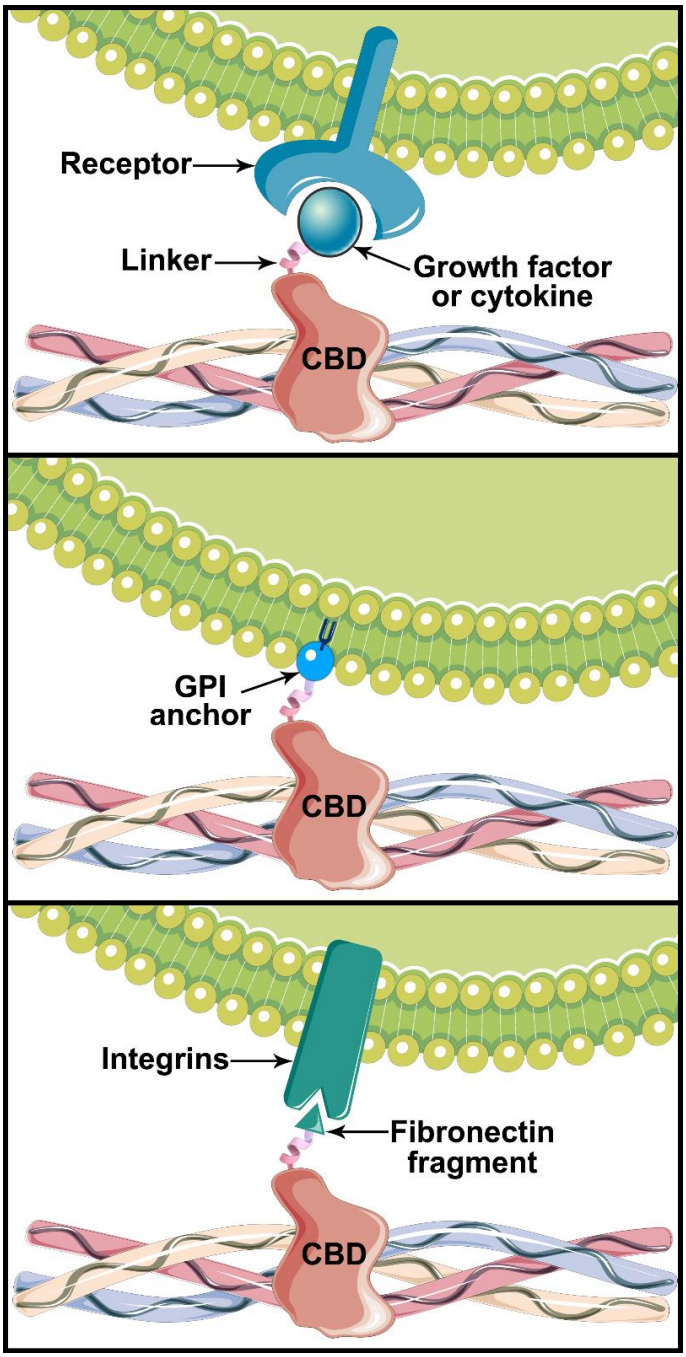

Figure 3.2. Biological effect of various chimeric proteins containing a collagen-binding domain (CBD). Collagen can be functionalized with growth factors or cytokines to stimulate cell proliferation or differentiation (A), cells can be engineered to provide collagen anchorage through a GPI-linked protein (B) and collagen can be decorated with cell-binding domains to promote cell adhesion (C).

3.5.1 Chimeric collagen-binding proteins for wound healing

Wound healing is a complex process initiated by an inflammatory phase (which involves the clotting cascade and the recruitment of neutrophils and macrophages), followed by a proliferative phase (characterized by a strong angiogenesis, fibroplasia, formation of granulation tissue and collagen deposition) and that ends with a remodeling phase[132]. Throughout this process, growth factors play crucial roles as chemoattractants (EGF, FGF-1/2, PDGF, TGF-β, SDF-1α)[133], [134], mitogens (EGF, FGF-1/2, HGF, PDGF)[133], [135] and promoters of angiogenesis (FGF-1/2, NGF-β, PDGF, TGF-β, VEGF)[136], [137]. All of these factors are released in a highly controlled manner for an optimal spatio-temporal distribution leading to the appropriate modulation of cell response[135]. Hence, providing exogenous growth factors may accelerate or improve the quality of the wound healing process, although the success of the approach greatly depends on the carrier used for their topical delivery[138]. One of the major drawbacks that limits the efficacy of bolus injections of growth factors for wound healing is their very short half-life in vivo[139], a problem that is sometimes addressed by daily administration of growth factors[137]. As an alternative, chimeric proteins corresponding to growth factors fused to CBD have been developed to overcome this limitation (Fig. 2A), so as to prevent the diffusion of the growth factors while maintaining their bioactivity.

3.5.1.1 EGF

It has been long known that the topical application of EGF may accelerate the rate of epidermal regeneration in the case of partial thickness skin wounds or chronic wounds, although repeated applications are required[12], [140]. In order to bolster EGF effect in vivo, several groups have engineered EGF fusion proteins displaying collagen-binding abilities.

One of the first CBD-containing fusion protein assessed in vivo corresponded to EGF linked to the WREPSFMALS peptide thanks to a 12 amino-acid-long linker[74]. This fusion protein, in contrast to the native soluble EGF, was shown to accumulate at the sites of inflammation of the colon in a nude mouse model of experimental colitis and to promote complete regeneration of the intestinal crypts after 3 days, when administered by enema[74]. The results indicated that one administration

of the fusion protein was sufficient to significantly improve healing when compared to native EGF and suggested that it bound to the collagen exposed at the site of injury site.

Ishikawa et al. developed a 46-kDa fusion protein with the CBD of Fibronectin (I_6-II_1-II_2-I_7-I_8-I_9) and EGF, namely FNCBD-EGF[141]. This protein had a very stable and lasting interaction with collagen coated dishes but it did not bind to a large extent when applied alone in a full thickness wound, most probably because too little collagen was exposed at the wound site[141]. However, the functionalization of collagen sponges with FNCBD-EGF showed very good retention of the growth factor up to 4 days after implantation in diabetic wounds[141]. Oppositely, a collagen sponge loaded with native EGF showed almost no retention, most probably due to EGF diffusion around the wound[141]. Similar results have been obtained with a collagen hydrogel applied on a diabetic wound[142], highlighting that the fusion protein can be carried within various devices. In addition, the authors demonstrated that this fusion protein could directly bind to injured vessels when administered intravascularly in a rabbit model of injured carotid artery[142].

However, other designs of chimeric proteins containing a CBD and EGF led to mitigated results. The EGF-CBD developed by Nishi et al., which contained the CBD of ColH (S2b+S3 domains)[143] did not exhibit the mitogenic effect one would expect from its EGF moiety. Similarly, Kim et al. designed several chimeric proteins of EGF fused to the CBD of *V. mimicus*, either at the EGF N- or C- terminus[144]. They observed mitigated responses in term of EGF bioactivity as one of the CBD-EGF fusion protein did not exhibit any mitogenic effect[144]. The cause(s) of these loss of activity could be numerous: EGF misfolding due to the presence of a particular CBD (as already observed with other tagged EGF if not properly refolded[145] or steric hindrance of the CBD in absence of a long-enough linker[146].

In summary, successful CBD-EGF chimeras have been developed for enabling the capture of the epidermal growth factor in several collagen-based biomaterials that were loaded prior to implantation, as well as in vivo capture following topical delivery without the need for a carrier. The latter approach may however be more promising for vascular injuries than for skin wounds, most likely due to differences in endogenous collagen exposure.

3.5.1.2 FGF

Fibroblast growth factors (FGF) are potent mitogens and promote neovascularization. In particular, acidic (aFGF or FGF-1) and basic (bFGF or FGF-2) FGF are the most studied members of this family. Their fusion to CBDs has been extensively assessed due to the potential benefits these chimeras might bear in the field of wound healing.

Andrades et al. showed that the fusion protein WREPSFMALS-bFGF significantly reduced the healing time of wounds in both normal and diabetic rats when topically applied in a collagen hydrogel[112]. Zhao et al. compared the bioactivity of the same fusion protein with that of the chimeric protein TKKTLRT-bFGF, by implanting a functionalized collagen membrane in rats[113]. They observed that the collagen membrane functionalized with TKKTLRT-bFGF was more cellularized and vascularized after 7 days than the membrane functionalized with WREPSFMALS-bFGF[113]. Given that the latter protein has a lower affinity for collagen than the former (Table I), they suggested that the higher the affinity of the fusion protein to collagen, the higher the retention and the more significant the vascularization and cellularization[113].

A number of studies have then been conducted by this group to demonstrate that the TKKTLRT-bFGF fusion protein combined with collagen membranes could prove useful to counteract many tissue damage situations, be it for bladder regeneration[147], full-thickness abdominal wall defect repair[148], uterine horn reconstruction (all in rat)[149] and extrahepatic bile duct regeneration in pig[150]. In all cases, very encouraging results were obtained; for example, 90 days after the implantation of a collagen membrane functionalized with TKKTLRT-bFGF in a rat uterine horn damage model, the pregnancy rate increased from 33%, when untreated, to 60% when treated with bFGF and to 87% when treated with TKKTLRT-bFGF[149].

Altogether, since the bFGF moiety naturally binds to collagen and gelatin[151], the studies highlighted that CBD-bFGF fusions further enhanced the biological effects of basic fibroblast growth factor when incorporated in a collagen scaffold. Importantly, the choice of the CBD must be appropriately made in the design of a collagen-binding fusion protein, as the affinity for collagen mediates the retention and release rate of the growth factor, in turn impacting its effects in vivo.

3.5.1.3 The VEGF/PDGF family

Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) strongly promote angiogenic activity, in particular during the proliferative phase of wound healing[152], [153]. The translation of VEGF to clinical use in regenerative medicine is however compromised by its propensity to induce vascular permeability, which can lead to systemic hypotension, edema and even heart failure[154]. This limitation prevents its delivery in diffusible form in both peripheral and cardiovascular applications, due to its systemic biodistribution[14], [155]. A chimeric protein corresponding to VEGF fused to a CBD may thus eliminate this caveat by limiting VEGF diffusion.

Ishikawa and colleagues developed in that endeavor the FNCBD-$VEGF_{121}$ fusion protein (where FNCBD refers to full length CBD, i.e., I_6-II_1-II_2-I_7-I_8-I_9, Fig. 1A). After injecting the chimera into the injured tibialis anterior muscle in mice, they observed that it was retained for more than 24 h at the injection site and promoted the proliferation of interstitial cells, as opposed to native $VEGF_{121}$ or $VEGF_{165}$[52]. Furthermore, in a mouse hindlimb ischemia model, the intramuscular injection of FNCBD-$VEGF_{121}$ did not promote the mobilization of endothelial progenitor cells in the blood after 4 days, contrary to $VEGF_{121}$ or $VEGF_{165}$[52]. Altogether the results suggested that FNCBD-$VEGF_{121}$ was successfully retained in the muscle through its CBD without any systemic effect.

More recently, Martino et al. developed two chimeric proteins corresponding to $VEGF_{121}$ and PDGF BB fused to the CBD they identified in PlGF-2. The two proteins were tested in a diabetic mouse model of full-thickness wounds[14]. In comparison with the native growth factors, both fusion proteins significantly accelerated the closure of the wound, improved the granulation tissue formation and enhanced its neovascularization after 10-15 days[14]. Moreover, in comparison with native VEGF, the application of the VEGF-CBD drastically reduced the vascular permeability of the vessels when applied on the mouse ear skin, further suggesting that this fusion protein allowed for the separation of the angiogenic role of VEGF from its hyper-permeability activity[14].

Similarly, Zhang et al. fused the TKKTLRT peptide to the N-terminus of $VEGF_{121}$ and demonstrated that the functionalization of a collagen membrane with this chimeric protein significantly enhanced the neovascularization of the membrane after two weeks, when implanted subcutaneously[118]. More, the injection of this fusion protein in a rat infarcted myocardium

significantly reduced the scar size, enhanced capillary growth and improved the cardiac functions 4 weeks after injection, when compared to native $VEGF_{121}$[118]. The fusion protein was also almost undetectable in the serum 3 to 6 h post injection, although it was still present in the infarcted zone. In stark contrast, native $VEGF_{121}$ had diffused into the circulation[118]. Gao et al. obtained similar results with a collagen membrane functionalized with the same fusion protein in a rabbit infarcted myocardium model[156], providing strong evidence that myocardial infarction could be treated with a cardiac patch made of CBD-VEGF-functionalized collagen.

Encouraging results were also reported for the treatment of full thickness skin wound in diabetic rats, be it by repeated injections of the same fusion protein at the site of injury[157] or via the application of a functionalized collagen membrane[158]. Further attempts were made, with very promising results, to treat extensive urethral defect in dogs[159] and full-thickness injury uterus in rats[160]. For example, 60 days after injection in a rat scarred uterus, the pregnancy rate at the scar site increased from 6% in the control group to 19% in the group treated with VEGF and to 50% in the group treated with TKKTLRT-VEGF[160].

Similarly, Akimoto et al. constructed a fusion protein with the murine $VEGF_{164}$ and the S3 domain of *C. histolyticum* ColH, which was tested in a rat dorsal-skin flap model[161]. The fusion protein significantly decreased the necrosis rate and promoted neoangiogenesis after 7 days, when injected subcutaneously[161], suggesting that the bioavailability of the growth factor was prolonged. However, in this particular study, the injection of native $VEGF_{164}$ did not show any effect on the necrosis rate, in disagreement with previous studies[162], [163].

Lin et al. developed the fusion protein TKKTLRT-PDGF BB to decorate a collagen membrane[43]. 4 days after subcutaneous implantation, the biomaterial significantly promoted the cell ingrowth and neovascularization, when compared to the membrane loaded with pristine PDGF BB[43]. Moreover, when employed to treat rabbit dermal ischemic ulcer, the functionalized membrane significantly enhanced the re-epithelialization of the wound, the formation of granulation tissue and the neovascularization 14 days after implantation[44].

Altogether, CBD-VEGF fusions may have solved the major limitation of VEGF regarding its clinical translation, that is, the separation of its angiogenic properties from its negative impact on vascular permeability. More, the collagen-binding fusion proteins of VEGF and PDGF can be

administered to enhance the repair rate and extent in various types of wounds, by increasing the bioavailability of the growth factors, when compared to their native counterparts.

It is here worth mentioning that the high-molecular weight isoforms of VEGF possess a heparin-binding domain that can be used to improve their retention[164], be it via interaction with endogenous heparan sulfate or with a heparinized biomaterial[165]. However, the interaction of VEGF with endogenous heparan sulfate may be less stable than those of CBD-VEGF with collagen, since the administration of the native growth factor do not yield the same effects, as observed in most of the above-mentioned studies that used $VEGF_{165}$ as control.

3.5.1.4 HGF

The hepatocyte growth factor (HGF, also named scatter factor) is an important cytokine in wound healing, given that it induces granulation tissue formation, promotes angiogenesis and accelerates re-epithelialization in vivo[166]. HGF synthesis and production are however complex insofar as they require the cleavage and processing of a single chain precursor by an enzyme, the HGF activator, leading to a mature heterodimer protein composed of the α and β chains[167].

Kitajima et al. engineered a 120-kDa chimeric protein corresponding to a shortened CBD derived from Fibronectin (I_6-II_1-II_2-I_7) fused to HGF and expressed it in Sf9 cells, an insect cellular platform that provides high levels of secreted proteins[109]. In vitro, the chimera stimulated the growth of human umbilical vein endothelial cells for more than 10 days, in contrast to the native growth factor that was rapidly internalized and degraded[109]. Seven days after the implantation of a collagen sponge in rats, the fusion protein significantly promoted neovascularization within the scaffold when compared to a sponge loaded with native HGF[109]. Moreover, the team reported that the density of blood vessels increased in a FNCBD-HGF dose-dependent manner[109], suggesting that the functionalization of the biomaterial could be fine-tuned for a specific application. Similarly to FNCBD-EGF, the fusion protein FNCBD-HGF did not accumulate in the wound to a larger extent than native HGF when applied topically in a dorsal full-thickness wound model[168], more likely due a limited exposure of endogenous collagen. This corroborates once again that the effectiveness of a collagen-binding fusion protein is bolstered when combined with the appropriate niche or carrier, such as a collagen hydrogel or a collagen membrane.

The FNCBD-HGF fusion protein was also tested in vivo by Ota et al. to treat a myocardial defect in pig, using a cardiac patch based on a collagen-containing urinary bladder matrix[169]. After 60 days, the functionalized patch significantly improved the recovery of the mechanical and electrophysiological functions of the myocardium when compared to a patch made of Dacron[169]. Of salient interest, Ohkawara et al. demonstrated that this protein accelerated the re-endothelialization of a rat carotid artery that had been injured by a balloon procedure. More precisely, re-endothelialization was observed when the protein was infused directly in the artery for 15 min, whereas infusion with native HGF did not exert such a healing effect[170]. However, this treatment significantly aggravated the hyperplasia of the neointima, most probably due to a higher local HGF concentration or a longer HGF half-life, which aggravated the stimulation of smooth muscle cells proliferation[170].

Altogether, although the production of recombinant HGF and HGF-derived chimeras is complex, the propensity of CBD-HGF fusions to enhance healing in vascular wounds has been demonstrated. Importantly, HGF potent mitogenic activity on smooth muscle cells requires their dosage to be tightly regulated, so as to mitigate hyperplasia.

3.5.1.5 Cell therapy

A significant improvement upon the localized administration of growth factors could be achieved by delivering specific cells at the site of injury and guiding them towards exposed endogenous collagen in vascular wounds.

This innovative concept was elegantly introduced by Tan and colleagues who engineered a fusion protein composed of the A3 domain of vWF and a glycosylphosphatidylinositol (GPI) anchor, a glycolipid naturally displayed at the cell membrane[171] (Fig. 2B). This glycosylated chimeric protein was produced in Chinese ovary hamster cells and used for the surface-functionalization of bone-marrow-derived endothelial progenitor cells (EPC). More specifically, EPCs were incubated with the vWF-GPI chimera which penetrated their lipid bilayer, thus providing them with a collagen-binding capacity. The CBD-displaying cells were then infused in the artery of a mouse suffering from a carotid injury. A significantly higher number of cells were incorporated at the site of injury, which led to an improved re-endothelialization of the artery when compared to the control

animals, in which the injected cells had their extracellular A3 domain cleaved from the GPI anchor prior to infusion[171].

A similar innovative approach has been developed by Shao and colleagues, who used a polypeptide containing both the collagen-binding sequence TKKTLRT and the cell-binding peptide EPLQLKM (the latter was identified by phage display and has a high specific affinity to bone marrow-derived mesenchymal stem cells, MSCs)[172]. Upon implantation in pig full-thickness wounds, collagen membranes functionalized with this polypeptide improved the healing rate, displaying numerous infiltrating cells and high blood vessel density, in stark contrast with the collagen membrane alone[172]. Although these results suggest that the polypeptide enhanced the capture of autologous MSCs at the wound site, further studies would be required to confirm the efficiency of this capture system.

Although cell-based therapy may still be controversial and in its early stages, CBD-cell fusions have proved to be effective in accelerating wound healing in animal models.

3.5.2 Chimeric collagen-binding proteins for bone regeneration

Bone regeneration is an intricate process regulated by numerous cytokines and growth factors. Similar to wound healing, these bioactive molecules play different roles, from chemoattraction (BMP, FGF, PDGF, VEGF, TGF-β)[173] and cell proliferation (FGF, IGF, PDGF, TGF-β)[173] to osteoinduction, i.e. differentiation of MSCs into mature bone cells (BMP, IL-11, TGF-β)[173]. Exogenous growth factors can thus be employed to functionalize scaffolds, such as demineralized bone matrix (DBM, made of spongy bone treated with HCl), in order to promote osteogenesis[33]. Some of these growth factors are already approved by the Food and Drug Administration (FDA) for bone regeneration purposes: for example, BMP2 can be administered to accelerate the healing of open tibial fractures[174]. However, the clinical translation of the other growth factors is seriously limited by the lack of control of their spatiotemporal release and the high doses that are required, which may lead to side-effects such as heterotopic bone formation or increased risks of cancer[33]. In this context, collagen-binding fusion proteins were shown to be very appealing to better control the localization of growth factors. In addition to being the main organic content of

bone, type I collagen is indeed the basis of several resorbable biomaterials that have already been approved by the FDA for bone repair[175], [176].

3.5.2.1 FGF

Within the context of bone regeneration, bFGF represents once again a potent mitogenic growth factor that is secreted by many cell types, including MSCs and osteoblasts[173], [177]. In the early phase of fracture healing, bFGF is associated with a rapid increase in the local amount of MSCs[173], [178]. Accordingly, exogenous bFGF is known for promoting callus formation and bone healing[173], [178].

In order to obtain a large quantity of chondrocytes, Du et al. cultivated MSCs in a 3D scaffold made of chondroitin sulfate and gelatin that was loaded with either both native TGF-β and bFGF or TGF-β and TKKTLRT-bFGF[179]. While half of the untagged bFGF was released from the scaffold within 1 day, the half-time of the release of its CBD-tagged version was almost 5 days. In fact, two distinct interactions control the release profile of both growth factors: on the one hand, the CBD interacts with gelatin and, on the other hand, bFGF and TGF-β bind to chondroitin sulfate owing to electrostatic interactions. The resulting functionalized 3D matrix proved to be an excellent cell niche for promoting the in vitro differentiation of MSCs into chondrocytes[179]. In addition to highlighting the potential of a CBD-FGF construct for bone regeneration, this study demonstrated how the combination of two distinct interactions can be exploited to potentiate the growth factors effect when embedded in an appropriate scaffold.

In an attempt to improve fracture healing, Saito et al. demonstrated the benefits of combining the administration of collagen powder with a CBD-bFGF fusion protein comprising the S2b+S3 collagen-binding domain of the ColH collagenase[180]. Four weeks after treatment of femoral fractures in mice, bigger callus volume and higher bone mineral content were produced, in comparison with the same collagen powder loaded with native bFGF[180]. In particular, Ueno and colleagues demonstrated that this treatment accelerated the bone union in a mice femur graft model, showing that a hard callus bridge was formed at the host/graft junction[181].

Moreover, Uchida et al. studied the impact of varying the affinity of the fusion protein for collagen by designing chimeric proteins corresponding to bFGF fused to various CBD versions, i.e. the

S2b+S3 domains or the S3 domain alone. Once combined with a collagen sheet and applied in a rat femur graft model, bFGF-S2b+S3 markedly enhanced bone formation in comparison with bFGF-S3[182]. This experiment confirmed that the affinity of the fusion proteins for collagen is a key factor in their efficacy, as S2b+S3 has higher affinity for collagen (see Table I).

In addition, this group demonstrated that the fusion protein bFGF–S2b+S3 can be combined to a large panel of collagen carriers, such as collagen powder derived from porcine skin[180], collagen sheet[183], demineralized bone matrix[183], demineralized bone powder[184], or even $(POG)_{10}$, a polymer which mimics collagen triple helix conformation[185].

In short, CBD-bFGF can be administered along with a large panel of collagen biomaterials to improve bone regeneration. However, the efficacy of the approach is largely driven by the affinity for collagen of this fusion protein.

3.5.2.2 The TGF-β family

TGF-β growth factors exert their role on many different cell types in the human body; in particular, they stimulate the migration and the proliferation of osteoprogenitor cells[173]. In addition, TGF-β1 was reported to promote early stage of osteoblast differentiation while inhibiting osteoinduction and mineralization at later stages[173].

Andrades et al. thus developed the fusion protein WREPSFMALS-TGF-β1 and demonstrated that its chemotactic activity was much higher than that of native TGF-β1, BMP2, BMP7 or even bFGF, by observing the migration of rat primary bone marrow cells cultivated in a collagen hydrogel[186]. More, the mitogenic activity of this fusion protein over rat MSCs was significantly higher than pristine TGF-β1, leading cells to form numerous colonies, similarly to BMP2 and BMP7 but much earlier in the differentiation process[186]. Conversely, calcium deposition, osteocalcin synthesis and ALP activity – all markers of osteogenic lineage – were also promoted in comparison with TGF-β1, although they did not reached the values induced by BMP2 or BMP7[186]. Overall, the chimeric collagen-binding TGF-β1 displayed a higher mitogenic activity than its native form, along with osteoinductive effects in the early phase of differentiation, whereas TGF-β1 had little to no effect. Similar results were obtained with human bone-marrow derived MSCs[187]. When the MSCs were implanted subcutaneously into diffusion chambers, the cells that had been pretreated

WREPSFMALS-TGF-β1 strongly promoted chondrogenesis, as cartilage accounted for 45% of the neoformed tissue, in comparison with the cells treated with native TGF-β1 (25%)[186], though none promoted bone formation. Claros et al. demonstrated that the complete osteoinduction of bone-marrow derived MSCs could be easily reached by switching WREPSFMALS-TGF-β1 to BMP2 on the later stages of differentiation, before implanting them subcutaneously into a diffusion chamber[188].

Attempts were also made to engineer a collagen-binding fusion with the TGF-β2 isoform, but no further in vivo characterization were made[189].

In summary, the CBD-TGF-β1 fusion proteins display significantly more potent chemotactic, mitogenic and osteoinductive activities than the pristine TGF-β1. The collagen-binding ability of the fusion proteins is more likely accountable for these effects, as it enhances the half-life of the chimeric proteins.

3.5.2.3 The BMP subfamily

The bone morphogenetic proteins (BMPs) belong to the large TGF-β family, they constitute a subfamily of key players in the regulation of osteoinduction. In bones, they are mainly synthesized by skeletal cells, such as osteoblasts[173]. BMP2 and BMP4 are striking examples of BMPs harboring osteoinductive properties: in vivo, they are known to promote the recruitment of MSCs and their differentiation into osteoblasts, resulting in ectopic bone formation[173]. In contrast, BMP3 is known for inhibiting osteoblast differentiation by interfering with osteogenic BMP signaling and is thus a negative regulator of bone density[190], [191]. It however promotes the proliferation of MSCs[192].

In an effort to promote bone regeneration, Chen et al. designed two BMP2 chimeric proteins C-terminally fused to a collagen-binding peptide, either WREPSFCALS or TKKTLRT. After 4 weeks, the subcutaneous implantation of DBM functionalized with WREPSFCALS-BMP2 in rats resulted in more ectopic bone formation in comparison with DBM loaded with native BMP2[193]. Similar results were observed with DBM functionalized with TKKTLRT-BMP2, leading to ectopic bone formation both in the center and on the outskirts of the implant. The research team reported that the regeneration was more homogeneous, with a higher bone density, when compared to the

DBM with pristine BMP2[119]. Unfortunately, a direct comparison of both fusion proteins was not performed by Chen and colleagues. On the same note, Visser et al. reported analogous results when implanting a collagen sponge that had been functionalized with the WREPSFMALS-BMP2 chimeric protein (400 – 10 000 ng) in the dorsal muscle of rats[77]. After 4 weeks, the WREPSFMALS-BMP2 loaded sponge not only induced the production of a higher quantity of bone, but also of a more mature bone than its native BMP2 counterpart, exhibiting numerous trabeculae, medullar cavities and blood vessels[77]. These two independent studies thus complement each other. They indeed demonstrate that the osteoinductive property observed by Chen et al. is mainly due to the BMP2 fusion proteins, rather than the DBM, a carrier known to have osteoinductive properties by itself[77]. Moreover, although it is generally admitted that BMP2 cannot induce osteogenesis under 460 ng in a rat model[194], Visser et al. showed that a quantity of collagen-binding BMP2 fusion protein below this threshold, i.e. 400 ng, was sufficient to induce bone formation, as opposed to the same quantity of native BMP2[77].

Chen et al. treated a critical-size rabbit mandible defect with a combination of DBM and TKKTLRT-BMP2 and showed that this treatment strongly promoted the healing of the mandible after 4 weeks. More precisely, neoformed bone occupied the main part of the defect site (80%), as opposed to the DBM with pristine BMP2 group (60%) and the negative control (<30%)[119], which suggests that this approach is promising in bone reconstruction. However, as DBM does not have sufficient mechanical compression strength, its application is limited to non-weight bearing bones. To overcome this problem, Zhao et al. studied a mineralized bone matrix (MBM) based on bovine spongy bone treated with H_2O_2, which has similar mechanical properties as untreated cancellous bone[120]. The team subcutaneously implanted MBM loaded with TKKTLRT-BMP2 in rats. After 7 days of implantation, a significantly higher ALP activity was noted, demonstrating that the cell ingrowth was enhanced, in comparison with MBM loaded with BMP2[120]. Similarly, after 60 days, more bone-like tissue was formed at the site of the MBM decorated with TKKTLRT-BMP2 implant[120]. Besides, Lai and colleagues studied the vertical bone regeneration by fixing titanium cylinders at the top of rabbit skulls, filling them with MBM and covering them with a collagen membrane being, functionalized with the TKKTLRT-BMP2 construct[195]. After 6 weeks, they observed that new bone formation was promoted not only from the surface of the native bone, but also from the superficial structures in contact with the collagen membrane[195],

suggesting that the functionalized membrane induced bone formation within the cylinder (such was not the case for the membrane loaded with pristine BMP2).

As an alternative to the collagenase-derived CBD, Martino et al. engineered two chimeric proteins corresponding to BMP2 and PDGF BB fused with the CBD of PlGF2[14]. Their topical delivery in a rat critical-size calvarial defect significantly increased bone tissue deposition (75% coverage of the defect) and the number of mesenchymal stem cells/pericytes, in comparison with wild type growth factors (<50% coverage of the defect)[14]. This experiment demonstrated that, even in the absence of carrier, the collagen-binding domain from PIGF2 may favor the recruitment of the fusion proteins by the animal extracellular collagenous matrix and promote bone regeneration.

The tethering of other members of the BMP family was also investigated. For example, Han et al. functionalized collagen sponges with WREPSMALS-BMP3 fusion protein and implanted them subcutaneously in rats [196]. After 4 weeks, in comparison with the controls (collagen sponges loaded with native BMP3), the retrieved implants exhibited significantly more calcium deposition, which increased in a BMP dose dependent manner[196]. However, the retrieved implants did not present any sign of bone formation or any increase in the ALP activity[196], a marker of the osteoblastic differentiation[191]. A comparison of these results with those of Visser et al. consistently corroborated the fact that BMP3 alone does not have the ability to promote osteogenesis, as opposed to BMP2. However, Han et al. reported that, when collagen sponges (or collagen-coated ceramic blocks) were functionalized with WREPSMALS-BMP3, their introduction in a rat calvarial defect did promote bone repair after 4 weeks (as did native BMP3 to a lesser extent)[196].

Another BMP member, BMP4, was also studied by Lu et al., who engineered a BMP4 chimeric protein containing the full-length CBD of Fibronectin (I_6-II_1-II_2-I_7-I_8-I_9). The chimera production was achieved in transgenic silkworm[197], a system that provides high level of protein expression, along with post-translational glycosylation[198]. A collagen-PLGA hybrid scaffold was functionalized with CBD-BMP4, seeded with MSCs to induce in vitro osteogenic differentiation for 24h. The scaffold was then implanted subcutaneously in rats[197]. After 4 weeks, calcium deposition was observed in the CBD-BMP4 group, in stark contrast with all controls (native BMP4, CBD alone or PBS)[197]. Similarly, osteogenic markers were strongly expressed in the CBD-

BMP4 group, but not in the others[197], suggesting that the differentiation of the cells was due to the long lasting action of CBD-BMP4 that had been retained within the scaffold due to its CBD moiety. These results were supported by Shiozaki et al., who injected as little as 100 ng of CBD-BMP4 in a mouse cranial bone defect: after 2 weeks, substantial ingrowth of new bone formation was observed. In particular, the ossification area of the defect was significantly improved (~58%), in comparison with the BMP4 group (~37%), the CBD group (~23%) and the control group (~19%). Interestingly, the authors showed that the CBD of fibronectin itself had a small positive effect upon induction of bone formation, leading to potential synergistic osteogenic effects with the BMP moiety[197]. In an effort to treat osteochondral defects, Mazaki et al. also proposed to combine chimeric BMP4 with a photo-cross-linkable gelatin scaffold to be loaded with bone marrow-derived stromal cells[199]. In a rabbit model, this implant led to the repair of subchondral bone via the generation of de novo articular cartilage-like tissue[199].

In brief, the chimeric CBD-BMP family can strongly promote bone formation or bone reconstruction in vivo, although each BMP family member has its own specificities. They have shown to be a promising treatment in several bone defects, when administered in a proper collagen carrier, which can be adapted to meet the mechanical properties required for each application.

3.5.2.4 Parathyroid hormone

As opposed to growth factors that have a localized mode of action, the parathyroid hormone (PTH) is a major systemic regulator of bone metabolism[173]. It is well established that repeated subcutaneous injections of PTH increases bone formation, which is why PTH is an effective treatment for osteoporosis[173]. However, its clinical application is limited, on the one hand due to its side effects (hypercalcemia and tumor risk) and on the other hand due to the necessity to perform repeated injections for optimal positive effects[200]. In an effort to limit PTH injections, Ponnapakkam et al. designed a fusion protein, $PTH_{1\text{-}33}$–CBD, that contained the CBD of ColH (S3 or S2b+S3), to be either injected intraperitoneally or delivered intravascularly. The rationale was to promote bone regeneration on the long term by increasing PTH half-life[201]. In a mouse model of chemotherapy-induced osteoporosis, a single dose of PTH–CBD administered before the chemotherapy treatment was sufficient to improve bone mineral density and blood ALP activity of the mice, bringing them closer to their normal threshold after 8 weeks[201]. Of salient interest, the

fusion protein that contained the fragment corresponding to the S2b+S3 domains did not exhibit the same bioactivity in vivo as that with the S3 domain only. More specifically, the chimera that contained the longest CBD had no positive effect on bone mineral density, although it improved blood ALP activity[202], most likely due to the higher affinity of the chimera for collagen (Table I). In ovariectomized rats, a well-described animal model for postmenopausal osteoporosis, the monthly subcutaneous injection of PTH-S3 promoted a significant increase (+14%) in the bone mineral density of the rats, as opposed to native PTH (temporary increase +5% after 14 daily injections), despite its clearance from serum after 12h[200]. In addition, there was no observed hypercalcemia in the PTH-CBD treated animals, suggesting that this side effect was reduced thanks to the collagen-binding ability of the fusion protein[200].

In summary the administration of CBD-PTH chimeric protein can extend the systemic effect of native PTH, hence reducing the need for repeated injections and associated risks. Interestingly enough, the fusion protein harboring the highest affinity for collagen was not the more efficient for this specific application.

3.5.2.5 Cell binding domain

The RGD sequence, originally identified in FN, has been long known to be recognized by integrins[203], [204]. By providing cell with anchorage, this interaction can promote their migration, differentiation and even their proliferation[204].

Visser et al. produced the peptide WREPSFMALSGRGDS containing the CBD derived from vWF and the RGD tripeptide[205] (Fig. 2C). They loaded collagen sponges with the native growth factor BMP2 along with this peptide, before implanting them intramuscularly in rats[205]. The BMP2-RGD combination significantly promoted the osteogenesis in the implant, which included bone trabeculae and mineralized matrix after only 14 days of implantation[205]. In comparison, the implant functionalized only with BMP2 did not promote trabeculae organization and presented almost no calcium deposition, due to the subfunctional quantity of BMP2 employed (300 ng)[205]. Similarly, the RGD peptide alone did not promote any osteogenesis, indicating that the BMP2-RGD fusion enhanced the recruitment of cells into the implant and promoted osteogenesis.

Similarly, Won et al. engineered a 47-kDa fusion protein containing the sequence TKKTLRT fused to the osteocalcin (OC) protein (a marker of the late stage of the osteogenic lineage) and to the FN fragment FN_{9-10}, known for its cell-binding ability, resulting in the fusion protein TKKTLRT-OC-FN_{9-10}[206]. They treated critically-sized rat calvarial bone defects with collagen-coated polycaprolactone (PCL) scaffolds functionalized with the fusion protein. After six weeks, these implants significantly improved osteogenesis, with bone covering more than 50% of the defect, in comparison with the collagen-coated PCL scaffold (~30%) or the negative control (<15%)[206]. Unsurprisingly, the effects were dose-dependent, as the best results were obtained with the greatest amount of engineered protein. These results, along with an in vitro study[207], suggested that the two fragments of the fusion protein, OC and FN_{9-10}, play a synergic role in the colonization of the biomaterial by MSCs and their osteoinduction[208].

In summary, collagen-binding fusion proteins displaying a cell-binding ability can significantly improve cellular ingression towards collagen biomaterials, hence resulting in better osteogenesis.

3.6 Conclusion

In this review, we have highlighted the therapeutic potential of chimeric proteins corresponding to growth factors fused to collagen-binding domains for wound healing and bone regeneration, when these chimeric proteins are combined to collagen-containing scaffolds or coatings. Indeed, in many in vivo studies, the fusion of a given growth factor to a CBD has led to a drastic improvement of its therapeutic effect when compared to that of the growth factor administered in its soluble form. This enhancement is more likely attributable to a prolonged bioavailability of the growth factor; may it be due to the fact that the growth factor is preserved from degradation or that it is present for a longer period of time and at a higher local concentration in the vicinity of its cell surface receptors. Of interest, the use of a chimeric CBD-GF may also limit its side-effects, as observed for several soluble growth factors, since it allows for their efficient sequestration at the site of injury. The best example of this beneficial effect is arguably that of VEGF for which its fusion to a CBD permitted to uncouple its angiogenic role from its hyper-permeability activity.

In most scientific articles collated in this review, researchers report the benefits of a given CBD-GF fusion protein when added to a single collagen-based scaffold. Despite all the promising results

presented therein, these data remain hard to interpret as a whole in order to establish the importance of the biophysical traits of the interaction between the CBD and the collagen-based scaffold (e.g. GF loading capacity, binding affinity and stability) upon therapeutic outcomes.

Indeed, very few are the studies reporting the amount of the chimeric protein bound to the scaffold or the impact of varying this quantity. On the same note, the effect of replacing a CBD by another is merely addressed. A significant amount of work thus remains to be done in order to better characterize the interactions occurring between the various types of collagen and the different collagen-binding domains identified so far. These experiments are of prime importance, not only to resolve the discrepancies between several values listed in Table I, but also as to provide the research community with a broader knowledge of these interactions. We believe this characterization, if exhaustive, would guide the design of novel tailored CBD-GF chimeras for optimal therapeutic effects.

3.7 Acknowledgments

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3.8 Reference

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CHAPITRE 4 PRODUCTION ET PURIFICATION DE PROTÉINES CHIMÈRES – FONCTIONALISATION D'UN SUBSTRAT DE COLLAGÈNE

Le CBD de la fibronectine a été choisi pour concevoir un adaptateur moléculaire (CBD-Kcoil), notamment en raison sa très forte affinité pour le collagène, dans le but d'immobiliser des facteurs de croissance sur un substrat de gélatine. Le substrat étudié est une surface de polystyrène recouverte de gélatine, qui constitue un modèle simple pour simuler la fonctionnalisation de biomatériaux. Plusieurs facteurs de croissance étiquetés avec une hélice alpha Ecoil ont déjà été développés dans notre laboratoire et j'ai eu l'occasion de développer un autre facteur de croissance (Ecoil-bFGF) pour compléter cette panoplie. Le substrat de gélatine a été fonctionnalisé successivement par CBD-Kcoil puis par un facteur de croissance Ecoil-GF, afin de former un complexe stable. Les propriétés de la fonctionnalisation de surface ont ensuite été analysés d'un point de vue cellulaire, en particulier concernant leurs effets mitogéniques et anti-apoptotiques. Ces résultats ont été soumis à *Acta Biomaterialia* sous le n°AB-16-1351.

4.1 Article 2 - The use of a chimeric collagen binding domain of fibronectin to recruit coil-tagged growth factors on gelatin-based biomaterials

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4.2 Abstract

In the field of tissue engineering, the tethering of growth factors in an oriented manner can enhance their activity and increase their half-life. We chose to investigate the capture of the basic Fibroblast Growth Factor (bFGF) and the Epidermal Growth Factor (EGF) on a gelatin layer, as a model for the functionalization of any collagen-based biomaterials.

Our strategy relies on the expression of a chimeric protein, that is, a Kcoil-tagged collagen-binding domain (CBD) of the human fibronectin, to be used as an adaptor. We proved that it has the ability to bind simultaneously to a gelatin substrate and any Ecoil-tagged recombinant growth factors via E/K coiled-coil complex formation. The tethering of the growth factors was characterized by ELISA and surface plasmon resonance-based biosensing. The bioactivity of the immobilized bFGF and EGF was evaluated by a human umbilical vein endothelial cells proliferation assay and a vascular smooth muscle cells survival assay. We found that the tethering of EGF preserved its mitogenic and anti-apoptotic activity. In the case of bFGF, its capture thanks to the Kcoil-CBD adaptor protein, modified its natural mode of interaction with gelatin.

Keywords— Collagen-binding domain; coiled-coil; growth factor; tethering; biofunctionalization

4.3 Introduction

Collagen is one of the most promising materials in the field of tissue engineering, given its outstanding biocompatibility, biodegradability, and safety[1]. Various formulations of biomaterials made of collagen have been developed, such as hydrogels, sponges and microparticles. Denatured collagen (i.e. gelatin) is often preferred to collagen, as it can be easily cross-linked to improve its mechanical properties and its resistance to proteolysis[4]. Many collagen-based biomaterials have already passed the regulatory approval, such as Orthoss®[176] and Neuragen®[209].

These collagen-based biomaterials can be loaded with growth factors (GF) to improve their healing potential, by enhancing cell adhesion and stimulating proliferation and differentiation. However, the lack of control over the release of the GFs represents a major drawback since the orchestration of their spatiotemporal delivery is very important for their optimal bioactivity[7], [8]. Recombinant

GFs fused to collagen-binding proteins or peptides have been engineered to decorate collagen biomaterials, by providing a stable but non-covalent bond with collagen. Several recombinant collagen binding domains (CBD) have been studied to tether growth factors, such as the short TKKTLRT[98] and WREPSFCALS[37] peptides, but most of them display low affinity towards collagen when compared to the CBD of the fibronectin, for instance[85], [106], [131]. The latter has already been employed for engineering fusion proteins of various growth factors such as EGF[141], VEGF[52], HGF[109] and BMP4[197] – but not bFGF – with very promising results both *in vitro* and *in vivo*.

Basic Fibroblast Growth Factor (bFGF) is a strong promoter of angiogenesis: it acts as a chemoattractant and mitogen for fibroblast and endothelial cells[210]–[212]. bFGF also supports the self-renewal of embryonic and induced pluripotent stem cells (iPSC)[213] and it is implicated in several differentiation pathways[214]. Similarly, Epidermal Growth Factor (EGF) stimulates cell proliferation, differentiation, and survival[215]. Native bFGF is known to bind to collagen (and gelatin) through ionic interactions[151], whereas EGF does not.

We here explore the potential of a chimeric protein, corresponding to the CBD of the fibronectin fused to a coil peptide (the Kcoil), as a versatile adaptor capable of capturing soluble GFs tagged with the complementary coil peptide (the Ecoil), on gelatin substrates (Figure 4.1). As a case study, bFGF and EGF were selected since their immobilization on cell culture substrates has been shown to potentiate their activity[216].

We employed the fusion protein Ecoil-EGF which was previously developed and characterized in our laboratory[22], and designed two other fusion proteins labelled with distinct coil peptides that bind to each other with great affinity and specificity[28]: the Ecoil peptide was fused to bFGF, while the CBD of fibronectin was fused to the Kcoil peptide (Figure 4.2A). After expressing and purifying the proteins, we evaluated their ability to bind to their partners, that is, gelatin and Ecoil-tagged proteins for CBD-Kcoil, and the Kcoil peptide and the bFGF receptor ectodomain (FGFR1) for the Ecoil-bFGF fusion protein. We also confirmed the bioactivity of the Ecoil-bFGF construct over human umbilical vein endothelial cells. At last, we evaluated the bioactivity of Ecoil-bFGF and Ecoil-EGF tethered to the gelatin substrate through CBD-Kcoil.

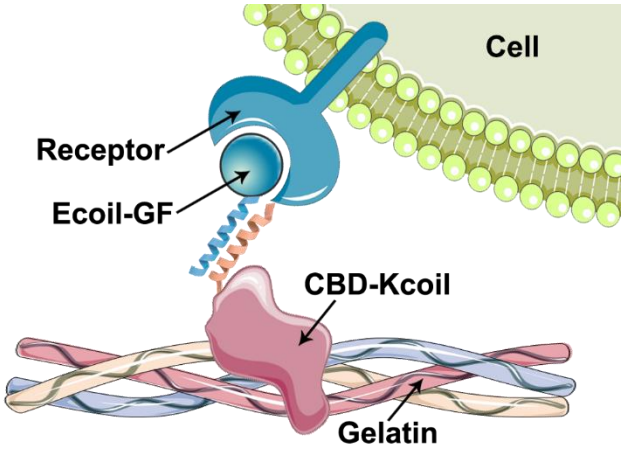

Figure 4.1. Schematic illustration of an Ecoil-tagged GF tethered in an oriented manner on a gelatin-coated surface that had been functionalized with CBD-Kcoil.
(This figure was prepared using tools from Servier Medical Art: http://www.servier.fr/)

4.4 Materials & Methods

4.4.1 Chemicals and reagents

Plasmids were purchased from GeneArt, Life Technologies (Burlington, ON). IPTG was from Inalco Pharmaceuticals (San Luis Obispo, CA). The TEV protease was a kind gift from Prof. J. Omichinski (Université de Montréal, QC). His-trap HP column, Mono S™ 10/100 GL column, ÄKTA-purifier UPC 10 system, CM5 sensor chips, HEPES buffered saline (HBS-EP) and amine coupling kit were purchased from GE Healthcare (Mississauga, ON). Spectra/Por® 6 dialysis membranes were purchased from Spectrum Laboratories Inc. (Rancho Dominguez, CA). Cysteine-tagged Kcoil peptides were synthesized by the peptide facility at University of Colorado (Denver, CO) as previously reported[217]. Gelatin from porcine skin (type A) was from Sigma Aldrich (St. Louis, MO). FGFR1-Fc and ELISA kits were purchased from R&D systems (Minneapolis, MN). Human umbilical vein endothelial cells (HUVEC), EBM-2 medium and supplements (EGM-2 single quote kit containing growth factors, fetal bovine serum (FBS), ascorbic acid, heparin and hydrocortisone) were purchased from Lonza (Walkersville, MD). A7r5 vascular smooth muscle

cells (VSMC) were from the American Type Culture Collection (Manassas, VA). DMEM/F12 and Pierce BCA Protein Assay Kit were obtained from ThermoFisher Scientific (Nepean, ON). Cells were cultured on 25 cm^2 CellBIND® flasks and 96-well CellBIND® plates from Corning (Corning, NY). Cell proliferation reagent (WST-1 tetrazolium salt) was purchased from Roche Diagnostics (Laval, QC).

4.4.2 Generation of the expression vectors

A first plasmid, pET-TRX-TEV-Ecoil-bFGF, was purchased from GeneArt. It contained the sequence coding for a NH_2-$(His)_6$-tagged fusion protein composed of the thioredoxin (TRX), followed by a tobacco etch virus cleavage site (TEV), the Ecoil peptide (i.e, $(EVSALEK)_5$), a spacer $(GGGS)_6$ and the bFGF sequence (Figure 4.2A).

Another plasmid, pET-CBD-Kcoil encoded for a NH_2-$(His)_6$-tagged fusion protein composed of the collagen-binding domain of the human fibronectin[109], [141], [218] from A260 to W599, followed by a spacer $(GGGS)_6$ and the Kcoil peptide, that is $(KVSALKE)_5$ (Figure 4.2A).

4.4.3 Expression of fusion proteins

Competent BL21(DE3) E. coli bacteria were transformed with the two plasmids and cultured at 37 °C in LB medium (in 4 L baffled flasks, 210 rpm) supplemented with ampicillin (100 µg/mL). Expression was induced by adding 0.7 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) when the optical density (OD) at 600 nm was c.a. 0.6. Cells were harvested 5 h later by centrifugation (15 min at 10,000 rpm). Cellular pellets were frozen at -20 °C and then re-suspended in 50 mL of lysis buffer (25 mM NaPO4, pH 7.4, 500 mM NaCl, 25 mM imidazole) and passed three times through a French press (1000 psi, SLM-Aminco® French Press) followed by sonication, for complete lysis. Ecoil-EGF was expressed and purified as previously reported[22].

4.4.4 Purification of Ecoil-bFGF protein

The cell lysate was clarified by centrifugation (60 min at 14,000 rpm, 4 °C) and the supernatant was loaded onto a 5-mL His-trap HP column charged with Ni^{2+}. The His-tagged fusion protein was eluted by applying a gradient from 25 mM to 500 mM imidazole (20 min at 5 mL/min). The elution fraction was then dialyzed against the TEV cleavage buffer (25 mM $NaPO_4$, pH 7.4, 125 mM NaCl,

1 mM EDTA, 10 mM β-mercaptoethanol) using a dialysis membrane (cut-off 8 kDa) overnight at 4 °C. The recombinant protein was subsequently treated with the TEV protease at an enzyme:substrate ratio of 1:80 for 2 h at room temperature and the cleavage mixture was directly injected onto a Mono S^{TM} 10/100 GL column that had been pre-equilibrated with 10 column volumes of equilibration buffer (20 mM $NaPO_4$, pH 7.4, 125 mM NaCl). Elution was then performed with a NaCl gradient (from 125 mM to 1 M, 20 min at 5 mL/min). The elution fraction was then passed through a 0.22 µm sterile filter and kept at -80 °C.

4.4.5 Purification of the CBD-Kcoil protein

The bacterial lysate was centrifuged (60 min at 14,000 rpm, 4 °C) and the pellet was re-suspended into denaturing buffer (25 mM $NaPO_4$, pH 7.4, 300 mM NaCl, 8 M urea, 1 mM β-mercaptoethanol) overnight. The supernatant harvested after centrifugation (14 000 rpm, 60 min, 4 °C) was diluted 1:1 in the lysis buffer (25 mM $NaPO_4$, pH 7.4, 500 mM NaCl, 25 mM imidazole). It was then loaded onto a 5-mL His-trap HP column charged with Ni^{2+}, previously equilibrated with a denaturing buffer: lysis buffer mix (1:1). The protein refolding was performed on the column (4 M to 0 M urea gradient for 120 min at 5 mL/min) and the protein was then eluted with 300 mM imidazole. The elution fraction was clarified through a 0.22 µm sterile filter and kept at -80 °C.

4.4.6 Surface plasmon resonance (SPR) assays

All kinetic experiments were performed at 25 °C using HBS-EP as running buffer at 100 µL/min on a Biacore T100 biosensor, with CM5 sensor chips. Sensorgrams were double-referenced prior analysis[219].

Gelatin was covalently immobilized using the standard ligand amine coupling procedure. Specifically, surface carboxylic acid groups were activated by the injection of a mixture containing 0.05 M NHS and 0.2 M EDC for 7 min, followed by the injection of gelatin at 0.1 g/L in HBS-EP for 7 min (at 10 µL/min). Blocking was performed using 1 M ethanolamine, pH 8.5 (7 min). CBD-Kcoil diluted in HBS-EP was injected at various concentrations for 60 s, followed by a 540 s dissociation period. Regeneration was performed between each cycle with 6 M guanidine hydrochloride.

Cysteine-tagged Kcoil covalent immobilization was performed as previously described[220]. Ecoil-bFGF was injected at 10 nM for 180 s, followed by a 180 s buffer injection. FGFR1-Fc (Recombinant Human FGF R1 beta Fc chimeric protein) was then injected for 180 s, followed by a 360 s buffer injection. Between each cycle, regeneration was performed by injecting 30 µL of regeneration buffer (100 mM acetate, pH 4, 2 M NaCl).

4.4.7 ELISA

Wells of a 96-well CellBIND® plate were pre-coated for 2 h with 100 µL of PBS with 0.1% gelatin. 100 µL of CBD-Kcoil diluted in PBS at various concentrations was then added for 1 h. 100 µL of Ecoil-bFGF or Ecoil-EGF diluted in PBS with 0.5% BSA were then added at various concentrations for 1 h. Between each step, three extensive washes were carried out with 200 µL of PBS-T (PBS with 0.05% v/v Tween® 20).

Tethered growth factor quantitation was performed using a direct ELISA by addition of 100 µL of biotinylated anti-bFGF or anti-EGF antibody (250 ng/mL, 1 h) followed by horseradish peroxidase (HRP)-streptavidin conjugate (1:40, 20 min) then 100 µl of substrate solution. Between each step, three extensive washes were carried out with 200 µL of PBS-T. The absorbance was read at 450 nm. Curve-fitting was performed with the GraphPad Prism 6 Software (GraphPad Prism Software Inc., San Diego, CA), using a Hill-type equation assuming a 1:1 stoichiometry of binding (the Hill coefficient was fixed at 1.0 for the sake of result interpretation).

For the stability assay, on the first day, a 96-well CellBIND® plate was pre-coated with gelatin, then every day, 4 wells were functionalized with CBD-Kcoil and Ecoil-EGF as described before. These wells always remained filled with 100 µL of either PBS or PBS + 10% v/v FBS and the medium was changed every day.

4.4.8 Proliferation assay

HUVECs were seeded at a density of 1000 cells/well in a 96-well CellBIND® plate. The cells were maintained in a humidified, 5%-enriched CO_2 incubator at 37 °C, using a minimal medium (EGM-2 complemented with 2% v:v FBS, 30 µg/mL gentamicin and 25 µg/mL ascorbic acid) and soluble Ecoil-bFGF at various concentration.

Cells were also seeded onto functionalized gelatin substrate wells, prepared as described in the preceding paragraph, and that have been extensively washed three times with 200 µL of PBS.

The medium was changed every other day. Cell metabolic activity was probed using a resazurin assay. Once rinsed with PBS, the wells were exposed to a mix of resazurin (100 µg/mL in MilliQ water) and culture medium (10:90 v:v) for 2 h. Fluorescence was read using a spectrophotometer (560 and 590 nm, for excitation and emission wavelengths, respectively). The experiments were repeated three times.

4.4.9 Survival assay

Rat vascular smooth muscle cells (VSMC) were seeded at a density of 2000 cells/well in a 96-well CellBIND® plate prepared as previously described and washed three times with PBS. The cells were plated in a humidified, 5%-enriched CO_2 incubator at 37 °C, using complete medium (DMEM/F12 complemented with 10% fetal bovine serum (FBS) and 30 µg/mL gentamicin). After a period of adhesion (24h), the cells were washed with PBS and the medium was switched to serum-free DMEM/F12 with 30 µg/mL gentamicin to induce the starvation.

The medium was changed every other day. Cell metabolic activity was probed as previously described. The experiment was repeated three times.

4.5 Results

4.5.1 Expression and purification of fusion proteins

The bacterial production and the subsequent purification of the chimeric proteins corresponding to bFGF and CBD, being N-terminally or C-terminally tagged with the Ecoil or Kcoil peptide, respectively (Ecoil-bFGF and CBD-Kcoil, Figure 4.2A) were assessed by SDS-PAGE. Under reducing conditions, the migration patterns of the affinity-purified chimeric proteins confirmed the high purity of the products and were consistent with their theoretical molecular weights, that is, 49.0 kDa for CBD-Kcoil and 39.8 kDa for TRX-TEV-Ecoil-bFGF, respectively (Figure 4.2B). However, the Ecoil-bFGF chimera (22.9 kDa) did not migrate to where expected since it was located above the 25 kDa marker. This behavior could be attributed to the Ecoil moiety, which

most likely affected the migration of the chimera, similarly to what had been already observed in the case of Ecoil-EGF[22].

The yields of these protein productions were of 1.1 mg and 1.9 mg of pure protein per liter of bacteria for Ecoil-bFGF and CBD-Kcoil, as determined by ELISA against bFGF and in a BCA assay, respectively.

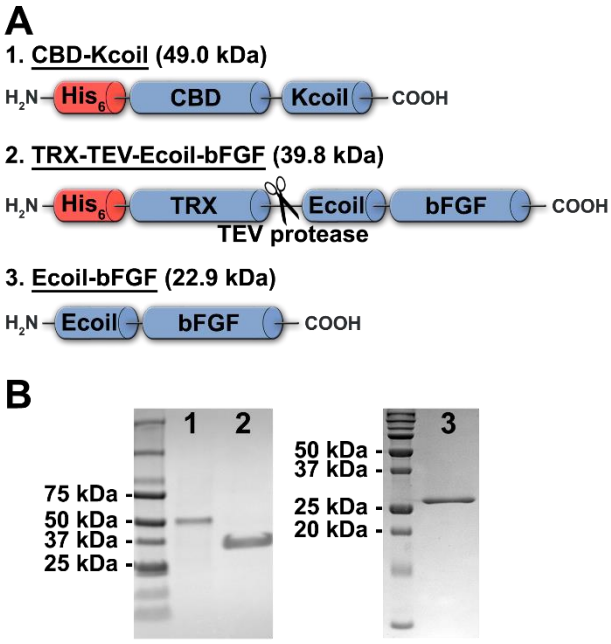

Figure 4.2. Structure, production and purification of the coil-tagged chimeric proteins used in this study.

(A) Structure of CBD-Kcoil (1) and TRX-TEV-Ecoil-bFGF before (2) and after (3) cleavage by the TEV protease.

(B) Recombinant proteins were analyzed by SDS PAGE under reducing condition and the gels were stained with Coomassie brilliant blue. CBD-Kcoil (1), TRX-TEV-Ecoil-bFGF (2) and Ecoil-bFGF (3).

4.5.2 Ecoil-tagged growth factors can be recruited on gelatin through CBD-Kcoil interactions.

In order to test the bioactivity of the CBD-Kcoil chimeric protein, that is, its ability to bind simultaneously to gelatin and Ecoil peptide, various concentrations of CBD-Kcoil (1 to 1000 nM) were incubated in the wells of 96 well-CellBIND® plates that had been pre-coated with gelatin. After extensive washing to remove unbound CBD-Kcoil, Ecoil-bFGF or Ecoil-EGF were added at various concentrations and washed. Enzyme-linked immunosorbent assays (ELISA) against bFGF or EGF were performed to quantify the amount of bound GF, and thus the formation of a gelatin/CBD-Kcoil/Ecoil-GF ternary complex. As shown in Figure 4.3, Ecoil-bFGF and Ecoil-EGF were indeed recruited through coiled-coil interactions in a dose-dependent manner. Of salient interest, the non-specific binding of Ecoil-EGF and Ecoil-bFGF over gelatin was hardly detected by ELISA in the range of the concentrations we studied.

The data were curve-fitted for the highest concentration of CBD-Kcoil (1000 nM) and led to a half-maximum effective concentration (EC_{50}) of 525 ± 25 pM, which is representative of the dissociation constant of the coiled-coil interaction between CBD-Kcoil and Ecoil-GF. This value is slightly higher than the previously reported values of K_D for the interaction between Kcoil and Ecoil-EGF (K_D = 185 pM)[221] possibly due to steric hindrance of the CBD moiety.

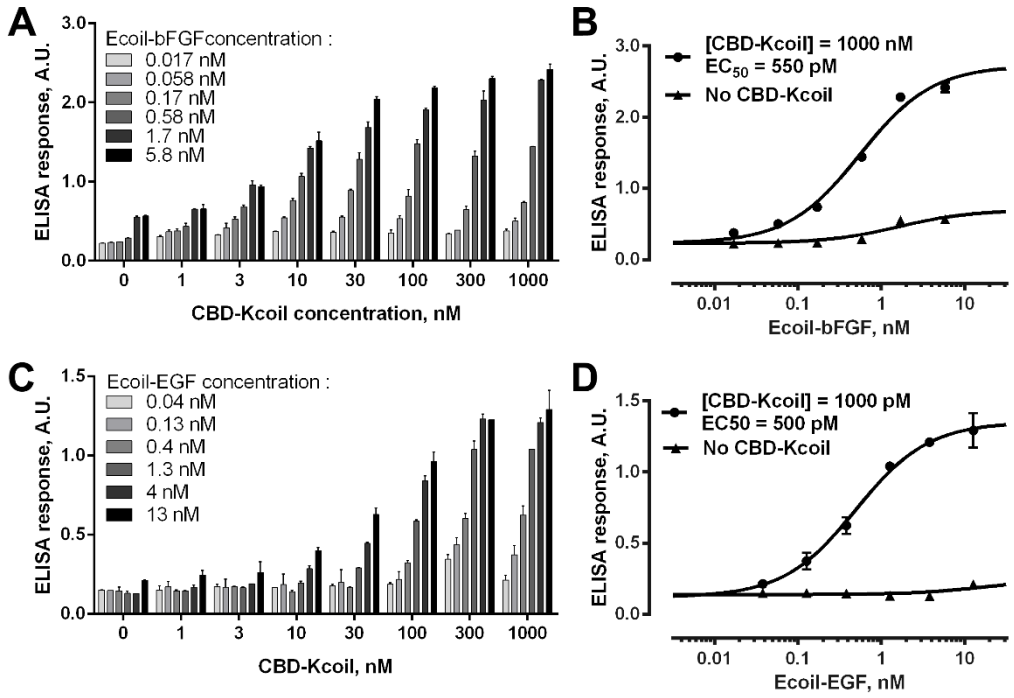

Figure 4.3. Tethering of Ecoil-bFGF (A) or Ecoil-EGF (C) on gelatin thanks to the CBD-Kcoil adaptor.

For data corresponding to [CBD-Kcoil] = 1000 nM, EC_{50} values for the E/K coiled-coil interactions were determined by fitting the ELISA data corresponding to Ecoil-bFGF (B) and Ecoil-EGF (D). Values are given as mean value ± standard deviation (n = 2).

4.5.3 Stability of the gelatin/ CBD-Kcoil/Ecoil-GF complexes

The interaction of CBD-Kcoil with gelatin was assessed by surface plasmon resonance (SPR). The protein was injected over a gelatin-coated chip at concentrations ranging from 10 to 100 nM. Control-corrected sensorgrams were indicative of a complex mode of binding as one can conclude from the visual inspection of their dissociation phase (Figure 4.4A). This deviation from a simple mode of interaction may be due to surface heterogeneity (that might have been favored by random gelatin immobilization via amine coupling procedure) or rebinding artefacts (e.g., if dissociated CBD-Kcoil was allowed to interact with immobilized gelatin rather than being eluted due to

diffusion limitations). The interactions were however well-fitted with a 1:1 stoichiometry model assuming a kinetically limiting CBD-Kcoil/gelatin complex rearrangement step after the initial interaction (two-step rearrangement model). This model was selected as it corresponds to a 1:1 stoichiometry and as it is consistent with a cooperative mode of interaction due to the modular nature of CBD that contains six distinct domains all required for full affinity[53], [55]. This led to an apparent thermodynamic dissociation constant, $K_{D,\,app}$ of 3.2 nM, indicative of a strong collagen-binding interaction. Similar results were obtained with an Ecoil-tagged CBD (data not shown), thus confirming that the Kcoil moiety did not participate to a large extent to gelatin binding. In comparison, native fibronectin has been reported to bind to type A gelatin with an apparent $K_{D,\,app}$ of 0.08 nM in an SPR assay[106]. This 40-fold difference in affinity is probably due to an avidity phenomenon, given the dimeric nature of native fibronectin.

The stability of the gelatin/CBD-Kcoil/Ecoil-EGF complex was then assessed in an experimental design mimicking that of cell culture. More specifically, ELISA were conducted in gelatin-coated wells on which CBD-Kcoil and Ecoil-EGF had been added and left in PBS or PBS + 10% FBS for several days (with daily renewal of the medium) (Figure 4.4B). Since the thermodynamic dissociation constant of the coiled-coil interaction is lower than that of the collagen binding interaction, the observed decrease of the amount of tethered EGF is mostly due to the dissociation of the CBD domain from the gelatin substrate. In PBS, the complex displayed an initial release on the first 24h and then stabilized around 65% of its initial value, whereas in PBS + 10% FBS, the complex dissociated with a half-life of approximately two days.

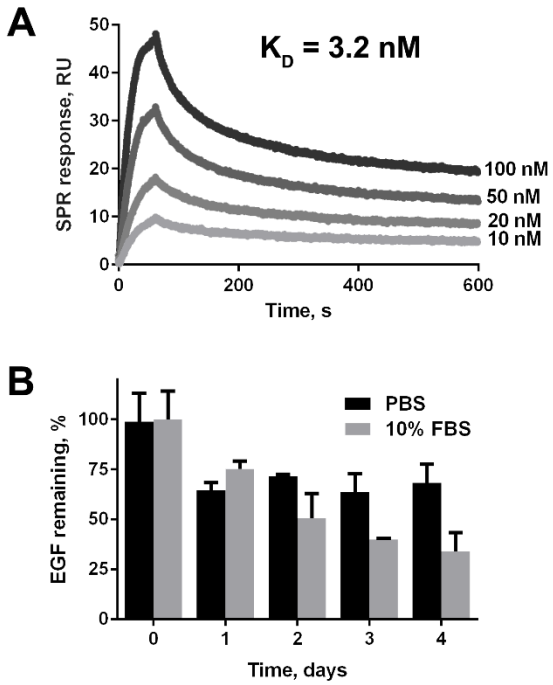

Figure 4.4. Characterization of the stability of the gelatin/CBD-Kcoil complex.

(A) Control-corrected SPR sensorgrams corresponding to the interaction of injected CBD-Kcoil with immobilized gelatin.

(B) ELISA against EGF was performed over gelatin-coated wells sequentially functionalized with CBD-Kcoil (300 nM) and Ecoil-EGF (10 nM). The wells were incubated either in PBS or PBS + 10 % FBS and the medium was changed every day.

Values are given as mean value ± standard deviation (n = 2).

4.5.4 Bioactivity of Ecoil-bFGF

HUVEC were cultivated in presence of either soluble Ecoil-bFGF or bFGF in the culture medium (1.1 pM to 3.5 nM) in a pristine CellBIND® plate. After four days of culture, the metabolic activity of the cells was assessed by a resazurin assay (Figure 4.5A). In solution, the Ecoil-tagged growth factor displayed a very similar behavior compared to native bFGF, with an EC_{50} close to 4 pM (equivalent to 0.07 ng/mL bFGF), a maximum of proliferation around 100 pM (equivalent to 1.7

ng/mL bFGF), followed by a decrease of its mitogenic activity at higher concentrations, in agreement with the literature[112], [113], [222].

Besides, the ability of Ecoil-bFGF being tethered via coiled-coil interactions to bind to its receptor was assessed by SPR. In the experiment shown in Figure 4.5B, 60 response units (RU) of Kcoil peptide were first immobilized on the SPR biosensor surface via a unique cysteine residue. 10 nM of Ecoil-bFGF were then injected on the Kcoil and mock surfaces to result in a 150 RU net accumulation (data not shown). The interaction between the Kcoil peptide and Ecoil-bFGF displayed a high affinity and stability, which is consistent with those we previously determined for the E/K coiled-coil interaction[27]. Afterwards, successive injections of FGFR1-Fc over immobilized Ecoil-bFGF unambiguously demonstrated that the captured growth factor was able to interact with its receptor ectodomain with high affinity (K_D = 1.8 nM). For the sake of comparison, Lin et al. showed that the injection of FGF-2 over immobilized FGFR1-Fc displayed a dissociation constant of 99 nM[223]. The 55-fold difference in affinity is most likely due to the dimeric nature of FGFR1-Fc that induced an avidity phenomenon in our experimental design only, i.e. when the bivalent receptor was in solution. For Ecoil-EGF, we have already demonstrated it is able to bind to its receptor when tethered via coiled-coil interactions[22].

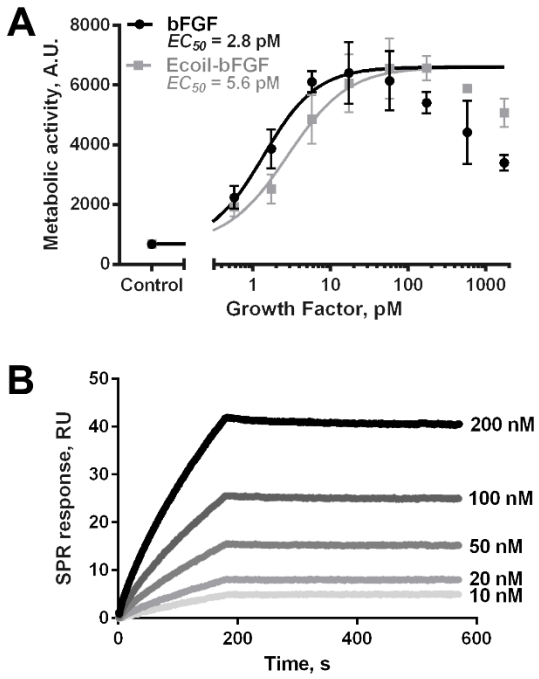

Figure 4.5. Characterization of the bioactivity of Ecoil-bFGF.

(A) HUVEC metabolic activity measurements, after 4 days in 2% FBS medium. Values are given as mean value ± standard deviation ($n \geqslant 3$).

(B) Control-corrected SPR sensorgrams corresponding to the interactions of injected FGFR1-Fc with Ecoil-bFGF immobilized via coiled-coil interactions on a Kcoil-coated chip.

4.5.5 Tethered Ecoil-bFGF and Ecoil-EGF promote cell proliferation

We then tested the ability of tethered Ecoil-bFGF to promote HUVEC proliferation. As can be seen in Figure 4.6A, as expected, CBD-Kcoil alone incubated at 300 nM on gelatin had no effect on HUVEC proliferation. In stark contrast, the addition of untagged or Ecoil-tagged bFGF (10 nM, followed by extensive washes) on pristine gelatin surfaces or on gelatin surfaces that had been incubated with CBD-Kcoil, resulted in a significant increase of cell growth, as deduced from the increase in metabolic activity (Figure 4.6A) and cell counts (data not shown). As a matter of fact, all of the conditions where bFGF or Ecoil-bFGF had been incubated over gelatin significantly promoted the proliferation of the cells, almost reaching the level of the positive control, that is, bFGF in solution (10 nM). These results highlight the fact that native bFGF naturally interacts with

collagen and in this case remains tethered on the gelatin layer after several washes, although it was hardly detected by ELISA (Figure 4.3). In an effort to dissect the differences in bFGF capture on gelatin, may it be through direct bFGF-gelatin interactions or via CBD-Kcoil, increasing concentrations of Ecoil-bFGF were incubated on gelatin that had been treated or not with 300 nM CBD-Kcoil. As can be seen in Figure 4.6B, the pre-incubation of CBD-Kcoil on gelatin reduced the proliferation of HUVEC for bFGF concentrations lower than 1 nM, when compared to Ecoil-bFGF alone, suggesting that CBD-Kcoil competed with Ecoil-bFGF for gelatin binding (one would have expected comparable levels of cell proliferation at low Ecoil-bFGF concentrations if such was not the case).

Of salient interest, the functionalization of gelatin with CBD-Kcoil (300 nM) and Ecoil-EGF (10 nM) significantly improved the proliferation of HUVEC (Figure 4.6C) and promoted the survival of VSMC in serum-deprived conditions (Figure 4.6D), when compared to all the controls. The levels of proliferation (or survival) were equal or slightly higher than the positive controls, that is, EGF in solution (10 nM).

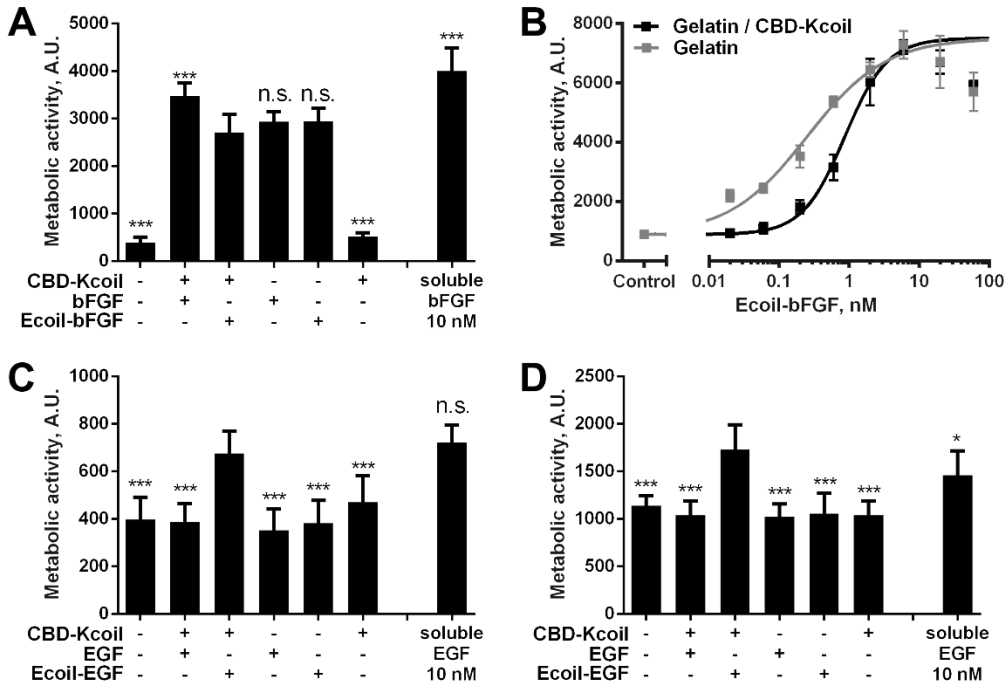

Figure 4.6. Bioactivity of Ecoil-bFGF and Ecoil-EGF tethered to collagen using a CBD-Kcoil adaptor.

(A) HUVEC metabolic activity measurements after 4 days in 2% FBS medium. Various combinations of proteins incubated over gelatin are presented. Soluble bFGF was used as positive control.

(B) HUVEC metabolic activity measurements after 4 days in 2% FBS medium. Ecoil-bFGF was incubated at various concentrations over gelatin pre-functionalized or not with CBD-Kcoil (300 nM).

(C) HUVEC metabolic activity measurements after 4 days in 2% FBS medium. Various combinations of proteins incubated over gelatin are presented. Soluble EGF was used as positive control.

(D) VSMC metabolic activity measurements after 4 days in serum-free medium. Various combinations of proteins incubated over gelatin are presented. Soluble EGF was used as positive control.

Values are given as mean value ± s.d. ($n \geqslant 9$). Statistical difference: n.s. stands for non-significant, * stands for $p < 0.05$ and *** for $p < 0.001$.

4.6 Discussion

A wide range of techniques have been developed for the tethering of growth factors to collagen scaffolds in order to provide a highly localized and long-lasting signaling. Among them, the random covalent grafting of growth factors has shown its potential[19], although in this case the bioactivity of the growth factors can be altered[23]. Another well-studied technique is the tethering of growth factors in a stable but non-covalent manner through specific affinity tags, such as biotin and streptavidin[26], coil peptides[29], DOPA-containing peptides[30] and heparin binding domains[31]. The use of chimeric proteins corresponding to growth factors fused to a collagen-binding domain has been intensively studied since type I collagen is the most abundant protein in the human body[35], meaning that these recombinant proteins could bind collagen biomaterials as well as endogenous collagen. Hence, the *in vivo* bioavailability of these chimeric proteins is significantly improved, as their diffusion is reduced.

We have here reported a system for the oriented capture of proteins over a gelatin or collagen substrate. The presented approach is versatile as it can be employed for the tethering of various kinds of proteins, in particular, we demonstrated that it perfectly suits the immobilization of growth factors. Moreover, this approach is modular as the use of an adaptor between the substrate (gelatin or collagen) and the protein (growth factors or other) provides several advantages. In particular, the strength of interaction between the protein and the substrate can be modulated by changing the affinity of the CBD moiety for the substrate. It is indeed possible to substitute the CBD of fibronectin for another CBD (for example the A3 domain of the von Willebrand Factor[67] or the CBD of the bacterial collagenase ColH[85]) or even a small collagen-binding peptide[14], without having to produce and purify other chimeric growth factors. Indeed, most growth factors are complex proteins that include several disulfide bridges and display a well-defined tertiary structure. It has already been established that the addition of a tag may negatively impact the bioactivity of the growth factor[22], therefore, modifying the adaptor removes all of the concerns about the bioactivity of the growth factor, synonym of an effort-intensive and time-consuming work. In the specific case of the E and K coils, the expression of numerous tagged chimeric proteins has been reported without any loss of bioactivity, such as growth factors (in particular EGF[22], VEGF[28] and bFGF, Figure 4.5A and 5B), the extracellular domains of various receptors (TGFβRII[220] and

TGFβIII[224]), and other proteins such as the alkaline phosphatase[225]. The specificity of the coiled-coil structure and the length of the coiled-coil complex appear to be key features resulting in an oriented tethering of growth factors at a sufficient (and possibly optimal) distance, which allows productive interactions with their cell-surface receptors. The affinity of the interaction between the growth factor and the substrate can also be modulated by varying the length[220] and the sequence of the coil peptide (Murschel et al. submitted) within the adaptor protein. Besides, this approach may also allow, if need be, the modification of the CBD adaptor chimera in order to add new functionalities, such as a recognition sequence for integrins in order to promote cell adhesion[226], [227].

The CBD we chose in this study is derived from human fibronectin (it includes all the modules from I_6 to I_9) and has an apparent affinity for gelatin of approximately 3 nM as determined by SPR (Figure 4.4A), which promotes its very stable attachment to gelatin-coated wells, with no release in PBS, and with a half-life of approximately 2 days in 10% FBS (Figure 4.4B). This is a direct consequence of the formation of a stable complex between gelatin and the CBD as observed in the dissociation phase of the SPR sensorgram, after an initial period of stabilization (Figure 4.4A). In combination with the very high affinity of the E/K coiled-coil interactions (ca. 500 pM, Figure 4.3B and 3D), the system we designed appears to be suitable for cell culture over several days without adding exogenous soluble growth factors. Moreover, this system is flexible since it is possible to vary the density of tethered growth factors by changing the concentrations of CBD-Kcoil and/or Ecoil-GF incubated (Figure 4.3A and 3C). This strategy of immobilization was successful in the case of EGF as a simple incubation of 10 nM of Ecoil-EGF allowed the capture of enough of this growth factor to get the same mitogenic and anti-apoptotic effects as those induced by 10 nM of soluble EGF renewed every other day (Figure 4.6C and 6D). These results are in accordance with what we observed for EGF tethered over other substrates on which Kcoil was immobilized in a covalent manner[29], [228]. Altogether, these results demonstrate the versatility and the endless possibilities of the coiled-coil system when combined with adaptor proteins, such as CBD.

However, in the specific case of bFGF, our results indicate that the use of a tethering system through an affinity tag for the capture of bFGF over a gelatin or collagen matrix must be analyzed with caution since bFGF displays an intrinsic affinity for these materials[151]. Indeed, our data

indicate that the interaction between our adaptor protein (CBD-Kcoil) and gelatin may be in direct competition with the interaction between bFGF and gelatin (Figure 4.6B). We would like to draw the reader's attention to the fact that the interaction between bFGF and gelatin could not be highlighted with an ELISA assay, as presented in Figure 4.3A. More specifically, in absence of CBD-Kcoil, the observed response levels were significantly lower (5-fold difference) than in presence of this adaptor protein. The most probable explanation is that the antibody against bFGF that was employed in the ELISA interacted with the residues – or close to the residues – involved in the interactions with gelatin. This point is worth-mentioning since it may change the interpretation of previously published results presented by other research groups. More specifically, even if the design and the use of chimeric proteins of bFGF fused to a CBD has shown its usefulness (and more particularly *in vivo*[143], [149], [181]), the interpretation of the results requires to have performed the appropriate controls in all of the experiments, namely, comparing the effect of the chimeric bFGF protein to those of pristine bFGF. We outline the fact that these controls are not systematically performed, probably due to negative results in ELISA assay[150], [226], [229].

4.7 Conclusion

We have developed a versatile strategy for the grafting of growth factors on collagen-based materials that involve a collagen-binding adaptor and coiled-coil interactions. We showed that our approach enabled the capture of both EGF and bFGF in a highly bioactive and stable manner. The modular approach we undertook may make this strategy amenable to the development of more complex scaffolds combining several growth factors with collagen-based biomaterials and thus pave the way to the development of novel approaches in the field of tissue engineering for the reconstruction of complex tissues or organs.

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4.9 References

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CHAPITRE 5 DISCUSSION GÉNÉRALE

Le concept d'immobiliser des biomolécules sur un biomatériau via un domaine de liaison au collagène est très prometteur (Chapitre 3) ; en plus des différents facteurs de croissances évoqués, d'autres entités ont déjà été attachées au collagène par des CBD, comme par exemple des anticorps dirigés contre des cellules cancéreuses[126], des protéines régulatrices du système immunitaire[100], des liposomes[230] ou même des nanoparticules afin d'imager le collagène *in vivo*[231]. La démarche consistant à créer un adaptateur moléculaire pour immobiliser n'importe quelle biomolécule permet la mise en place d'une **plate-forme modulaire** de fonctionnalisation du collagène. L'utilisation de l'interaction superhélice E/K, couplée à la très forte affinité du CBD de la fibronectine pour le collagène (K_D = 3 nM), rend cette plate-forme particulièrement **stable** et adaptée à une fonctionnalisation sur plusieurs jours. En outre, l'interaction superhélice peut être facilement modulée en modifiant le nombre d'heptades[220] ou en introduisant des modifications dans la séquence des hélices alpha (Murschel et al., submitted).

Par ailleurs, le développement de protéines de fusion comportant des étiquettes Ecoil est considérablement simplifié par rapport au développement de protéines fusionnées avec le CBD de la fibronectine, notamment à cause de la taille importante de ce dernier (39 kDa) et des nombreux ponts disulfures (12) qu'il contient. En particulier, il est raisonnable de penser que l'ajout de l'étiquette Ecoil (4 kDa) à ces protéines ne modifie pas substantiellement le rendement de production, ni la bioactivité de ces protéines chimères par rapport à leur forme native, que ce soit en cellules animales[22] ou en bactéries (Chapitre 4).

En outre, la plate-forme de fonctionnalisation que nous avons développée est particulièrement simple d'utilisation, dans la mesure où il y a peu d'étapes et qu'elle ne prend que quelques heures. Il serait également envisageable de fonctionnaliser la surface de gélatine en une seule étape, où les facteurs de croissance étiquetées (Ecoil-GF) et l'adaptateur moléculaire (CBD-Kcoil) seraient pré-mélangés, ce qui simplifierait encore le processus. Par ailleurs, il n'y a aucune réaction chimique, réactions qui nécessitent parfois des conditions opératoires difficiles (pH extrêmes ou solvant organique) ou des réactifs toxiques pour les cellules (glutaraldéhyde par exemple).

Les avantages liés à l'immobilisation de facteurs de croissance n'ayant aucune affinité pour le collagène sont très clairs, comme le démontrent les expériences menées avec Ecoil-EGF, qui confère à la surface des propriétés mitogéniques et anti-apoptotiques (Chapitre 4). Bien que ces avantages soient nettement moins évidents à observer dans le court terme avec les autres facteurs de croissances, comme c'est le cas pour Ecoil-bFGF, il est possible qu'une expérience sur une plus longue période et/ou dans un milieu de culture plus concentré en sérum ait montré une différence significative. En effet, l'interaction non-spécifique entre le bFGF et la gélatine est probablement moins stable que l'interaction de liaison au collagène du CBD. Cette hypothèse trouve echo dans les résultats des expériences de fonctionnalisation de biomatériaux en collagène avec des protéines chimères de la forme CBD-bFGF (Chapitre 3), et qui induisent un effet bénéfique par rapport au bFGF natif.

Il est clair que la stratégie développée dans les travaux de recherche exposés ici demanderait un certain effort pour passer à l'étape clinique, notamment du fait qu'elle implique plusieurs protéines non naturelles, qui doivent chacune prouver leur innocuité. Cependant, certaines protéines de fusion sont déjà autorisées par les autorités de régulation dans le cas d'applications bien précises, comme par exemple la fusion entre le facteur de coagulation VIII et le fragment Fc (d'un anticorps) ou encore entre le facteur IX et l'albumine, ce qui tend à prouver que ce n'est pas impossible.

L'idée sous-jacente à la fonctionnalisation de surface est, avant tout, de stimuler les cellules de l'hôte pour induire une réponse biologique : elle est donc potentiellement beaucoup plus simple à mettre en œuvre (voire plus économique) que n'importe quelle stratégie de délivrance cellulaire, qui implique d'avoir une certaine compatibilité, notamment au niveau du complexe majeur d'histocompatibilité. Par ailleurs, le risque d'induire un cancer par cette méthode est faible, en particulier lorsque peu de facteurs de croissance sont utilisés, par exemple dans le cadre d'une fonctionnalisation stable. Cependant, il est tout à fait imaginable à moyen terme de combiner ces deux approches, avec des cellules spécifiques incluses dans un biomatériau à base de collagène, lui-même fonctionnalisé pour promouvoir la survie ou la prolifération de ces cellules. Un bel exemple serait l'implantation de précurseur neuronaux pour le traitemet de maladies dégénératives comme la maladie de Parkinson.

CHAPITRE 6 CONCLUSION ET RECOMMANDATIONS

Les travaux de recherche présentés ont contribué à l'avancement des connaissances dans le domaine de la médecine régénératrice d'une part grâce à la publication de l'article de revue, qui compile l'ensemble des travaux effectués dans le domaine des CBD. En effet, aucune revue de littérature publiée ne détaillait jusque-là l'ensemble de la famille des CBD en les caractérisant et en répertoriant les différentes protéines de fusion déjà construites.

D'autre part, nous avons pu mettre en place et caractériser de manière poussée les propriétés biophysiques d'une plateforme de fonctionnalisation de la gélatine à l'aide de l'interaction superhélice « coiled-coil ». Une telle caractérisation permet non seulement de mieux comprendre 1) l'interaction entre le CBD et la gélatine et 2) l'interaction superhélice entre deux protéines de fusion, mais également 3) l'interaction entre les facteurs de croissance et la gélatine.

Cette stratégie de fonctionnalisation pourrait à l'avenir être améliorée en analysant les différences qu'il peut exister entre un substrat en collagène et en gélatine, ou encore entre un substrat en 2D et en 3D (une éponge de collagène par exemple). Par ailleurs, d'autres facteurs de croissance pourraient être testés, comme Ecoil-VEGF qui est une protéine dimère (bien que le VEGF natif se lie probablement déjà au collagène) ou d'autres biomolécules étiquetées avec Ecoil, comme des anticorps.

Le CBD de la fibronectine a été choisi car il possède une forte affinité pour le collagène, cependant, il est envisageable d'étudier d'autres CBD pour moduler cette interaction. Par exemple, des répétitions successives de peptides se liant au collagène (comme TKKTLRT) pourraient faire preuve d'une affinité comparable, (voire supérieure grâce au phénomène d'avidité), mais avec une taille plus modeste.

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